# Role of Aldosterone in Obesity-Induced Endothelial Dysfunction

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Universität Zürich

von

Nicola Schäfer

aus

Deutschland

Promotionskomitee:

Prof. Dr. François Verrey (Vorsitz)

PD. Dr. Christian M. Matter

Prof. Dr. Carsten Wagner

Prof. Dr. Thierry Pedrazzini

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# 1 Summary

The steroid hormone aldosterone plays a crucial role in cardiovascular disease as demonstrated by the fact that antagonism of the mineralocorticoid receptor (MR), which is the receptor of aldosterone, decreases atherosclerosis by reducing inflammation and oxidative stress. Obesity is a chronic inflammatory disease that is associated with the development of endothelial dysfunction, the initial step in the development of atherosclerosis. Interestingly, obesity is characterized by increased plasma aldosterone levels.

The aim of this thesis was to characterize the role of aldosterone and the endothelial MR signaling in the development of obesity-induced endothelial dysfunction.

To study the role of endogenous aldosterone in this context, we exposed 6-weeks old male *C57BL/6* mice to a normal chow or a high-fat diet (60% lard). The high-fat diet was administered alone or in combination with the MR antagonist eplerenone (200mg/kg/day). After 14 weeks of diet, obesity-induced impaired glucose tolerance, white adipose tissue inflammation and endothelial dysfunction were attenuated by eplerenone. Analyses of freshly isolated aortic endothelial cell mRNA revealed that eplerenone administration regulates beneficially obesity-induced expression of oxidative stress-modulating genes such as NADPH oxidase subunits p22phox and p40phox as well as superoxide dismutase (SOD)-1 and-3. Interestingly, eplerenone did not alter the mRNA expression of cyclooxygenases-1 (COX-1) and prostacyclin synthase that produce vasoconstricting prostanoids in obesity. To investigate the role of endothelial MR, we exposed endothelial-selective MR knockout (*EC MR KO*) mice to a high-fat diet or infused them with exogenous aldosterone (50µg/kg/d). Obesity- as well as aldosterone-induced endothelial dysfunction was entirely prevented by endothelial MR ablation. A similar protection was observed by COX inhibition with indomethacin, underlining the importance of increased endothelial COX-1 and prostacyclin synthase expression in obesity. Experiments with aldosterone-treated *EC MR KO* mice revealed that aldosterone induces expression of prostacyclin synthase mRNA in an endothelial MR-independent manner in aortic endothelial cells. We suggest that this increase in prostanoid-producing enzyme by aldosterone contributes critically to obesity-induced endothelial dysfunction. In addition, aldosterone modulates the expression of p22phox and SOD-1 in an endothelial MR-dependent manner. These aldosterone and endothelial MR-mediated changes related to oxidative stress might be the key events leading to obesity-induced endothelial dysfunction.

We show that genetic loss-of-function of the endothelial MR tips the balance to anti-oxidative side in aortic endothelial cells, resulting in attenuated endothelial dysfunction in obesity and conclude that the endothelial MR plays a crucial role in obesity-induced endothelial dysfunction.

# 2 Zusammenfassung

Das Steroidhormon Aldosteron spielt eine essentielle Rolle bei der Entstehung kardiovaskulärer Erkrankungen und aktiviert durch seine Bindung den Mineralokortikoidrezeptor (MR). Antagonismus des MR vermindert die Entwicklung von Arteriosklerose durch Reduktion von Endzündungen und oxidativem Stress. Fettleibigkeit ist ein chronisch entzündlicher Zustand, der mit der Entstehung von endothelialer Dysfunktion, dem initialen Schritt in der Entwicklung von Arteriosklerose, assoziiert wird. Interessanterweise sind erhöhte Plasma-Aldosteron-Werte charakteristisch für Fettleibigkeit.

Das Ziel dieser Doktorarbeit war es, die Effekte von Aldosteron und endothelialer MR Aktivierung bei der Entstehung von Fettleibigkeits-assoziierter endothelialer Dysfunktion zu analysieren.

Um die Rolle von endogenem Aldosterone in diesem Kontext zu untersuchen, haben wir sechs Wochen alte männliche *C57BL/6* auf eine normale Diät und eine Fett-Diät gesetzt. Die Fett-Diät wurde mit und ohne MR Antagonist Eplerenon (200mg/kg/Tag) verabreicht. Nach 14 Wochen Diät wurde sowohl die Adipositas induzierte verschlechterte Glukose Toleranz, sowie die Entzündung im weißen Fettgewebe, als auch die endotheliale Dysfunktion durch Verabreichung des Eplerenons vermindert. Die Analyse frisch isolierter Aorten-Endothelzellen-mRNA zeigte, dass sich eine Verabreichung von Eplerenon vorteilhaft auf die Expression von Enzymen auswirkt, die mit Adipositas assoziiert sind und oxidativen Stress modulieren. Dazu gehören die NADPH-Oxidase-Untereinheiten p22phox und p40phox, aber auch Superoxiddismutase (SOD) 1 und 3. Interessanterweise beeinflusst Eplerenon nicht die Expression von Cyclooxygenase (COX-1) und Prostacyclin-Synthase, welche bei Fettleibigkeit gefässverengende Prostanoide produzieren. Um die Rolle des endothelialen MR zu untersuchen, haben wir endothelspezifische MR Knock-Out-Mäuse (*EC MR KO*) auf eine Fett-Diät gesetzt oder sie zwei Wochen lang mit exogenem Aldosterone (50µg/kg/Tag) behandelt. Fettleibigkeit- und Aldosteron-induzierte endotheliale Dysfunktion wurde durch die Entfernung der endothelialen MR Expression vollständig verhindert. Ein ähnlicher Schutz vor endothelialer Dysfunktion konnte durch Verabreichung des COX-Inhibitors Indometacin erreicht werden. Dies unterstreicht die Bedeutung der erhöhten COX-1 und Prostacylin-Synthase Expression in Aorten-Endothelzellen bei Fettleibigkeit. Experimente mit Aldosteron-behandelten *EC MR KO* Mäusen ergaben, dass Aldosteron die Expression von Prostacyclin-Synthase auf eine MR-unabhängige Art in Aorten-Endothelzellen induziert. Wir vermuten, dass der von Aldosteron ausgelöste Anstieg des Prostacyclin produzierenden Enzymes maßgeblich zur Entstehung der Fettleibigkeit-induzierten endothelialen Dysfunktion, beiträgt. Außerdem moduliert Aldosteron die

Expression von p22phox und SOD-1 durch Aktivierung des endothelialen MR. Diese durch Aldosteron und endothelialem MR ausgelösten Änderungen, die mit oxidativem Stress assoziiert werden, spielen eine Schlüsselrolle in der Entwicklung Fettleibigkeit-induzierter endothelialer Dysfunktion.

Wir konnten zeigen, dass der Funktionsverlust des endothelialen MR einen antioxidativen Zustand der Aorten-Endothelzellen begünstigt und dadurch zu einer verminderten endothelialen Dysfunktion bei Fettleibigkeit führt. Daraus schließen wir, dass der endotheliale MR eine massgebliche Rolle bei Adipositas-induzierter endothelialer Dysfunktion spielt.

# 3 Introduction

## 3.1 Obesity

For many centuries, obesity was regarded as a symbol of wealth, healthiness and beauty. But over the years it has been recognized that immense corpulence can be associated with various life-threatening diseases.

Nowadays, obesity is defined as a condition where body fat has accumulated to an extent that has adverse effects on health, leading to reduced life expectancy and/or increased health problems[1]. A measure that defines obesity is the body mass index (BMI; *BMI = kilograms / meters*$^2$)[2]. It compares weight and height and defines people as overweight if their BMI is between 25 kg/m$^2$ and 30 kg/m$^2$, and obese when it is greater than 30 kg/m$^2$.

Obesity is most commonly caused by a combination of genetic susceptibility and individual behavior as excessive food intake and lack of physical activity (Figure 1). Especially abdominal obesity is an important risk factor for development of obesity-associated diseases[3-4].

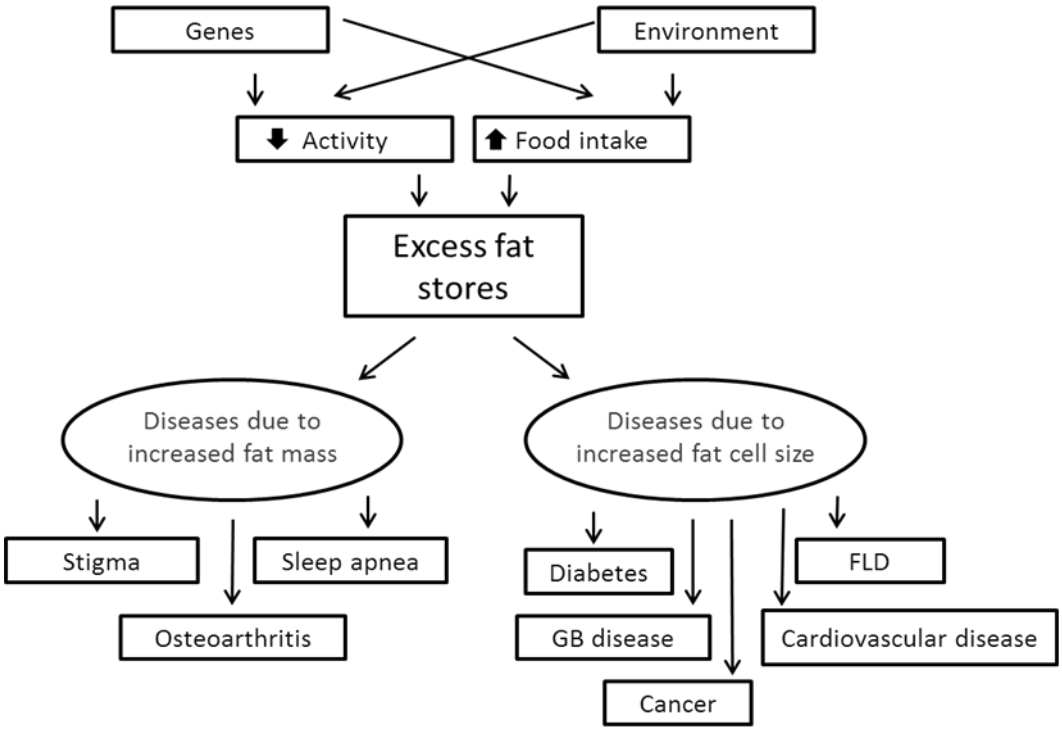


**Figure 1. The pathology of obesity produces several health-related problems.** These health problems can be attributed to increased mass of fat or increased release of peptides from enlarged fat cells. *GB* gallbladder, *FLD* fatty-liver disease. (Figure adapted from[5].)

The diseases caused by obesity and that arise from increased fat stores can be classified into two categories. The first class of disease is consequence of the accumulated fat mass itself like respiratory complications during sleep (sleep apnea), osteoarthritis and the stigma of being obese as the environmental reactions to the increased body size. The second category arises from metabolic changes that are associated with an increased fat mass as diabetes mellitus type 2, gallbladder disease, hypertension, cardiovascular disease, fatty-liver disease, and some forms of cancer associated with overweight (Figure 1)[5-6].

Some of these obesity-favored medical disorders can be summarized, called metabolic syndrome[7] that is characterized by any of the following factors[8]:

- raised fasting plasma glucose and insulin resistance
- elevated low-density lipoprotein-triglycerides (LDL-Tg)
- reduced high-density lipoproteins (HDL)
- raised blood pressure
- pro-inflammatory state
- vascular dysfunction
- pro-thrombotic state

All of these factors, implicated in obesity, contribute to the genesis of cardiovascular diseases as atherosclerosis.

### 3.1.1 Obesity-induced insulin resistance

With the increasing occurrence of the metabolic syndrome in the industrialized world, more attention has been drawn to the adipose tissue. Two different types of types of adipose tissue are known: the brown adipose tissue (BAT) and the white adipose tissue (WAT). Whereas the primary function of the BAT is the control of body temperature, the WAT is traditionally regarded as a lipid and energy storage organ that releases and deposits fatty acids.

Insulin is a pancreatic hormone that regulates carbohydrate and fat metabolism in the body by enhancing glucose uptake from the blood in adipocytes, being the major cell-type of the WAT, liver and muscle. Furthermore, insulin inactivates the hormone-sensitive lipase, which is responsible for the breakdown of triacylglycerol molecules and mobilizes energy stores in the tissue. Increased fasting plasma insulin and an enhanced insulin response to an oral glucose challenge is one major characteristic of obesity[9]. Interestingly, overweight individuals with most of their fat stores in the abdominal depots suffer of greater metabolic consequences than similar obese subjects with fat stores predominantly in subcutaneous sites[10-11].

This is due to the anatomical site of the visceral WAT that drains via the portal venous system. Thus, the liver is exposed to an increased repertoire of FFA, glycerol and lactate that is enhancing hepatic gluconeogenesis[7,12].

The different fat depots within the body vary in their responsiveness to hormones that counteract insulin action and are involved in the regulation of fat breakdown, called lipolysis. Because of higher expression of the β1- and-β2-adrenergic receptors[12-13] as well as of glucocorticoid receptor[14], the abdominal WAT shows an increased lipolytic response to the neurotransmitter noradrenalin and the glucocorticoid cortisol that enhance the exaggerated release of free fatty acids (FFA) from adipocytes into the circulation (Figure 2)[15-16]. The increased FFA in obesity induces hepatic gluconeogenesis, inappropriate glucose release and in turn, impaired hepatic glucose tolerance. This contributes to generation of raised circulatory insulin concentrations, called hyperinsulinemia. This post-hepatic insulin delivery is increased in upper body obesity resulting in increased peripheral insulin concentrations and in turn, due to downregulation of the insulin receptors in adipose tissue and muscle, insulin resistance. Initially, the β-cells of the pancreas compensate for this process by producing more insulin. In time, there is failure of the β-cells and followed by an increase of circulating blood glucose concentration, called hyperglycemia and in turn, type 2 diabetes. Hyperinsulinaemia and insulin resistance correlate with a dyslipoproteinaemic state and contribute to a obesity-associated plasma lipid profile that is characterized by elevated fasting plasma triglyceride concentration, reduced high-density lipoprotein (HDL)–cholesterol, elevations of cholesterol and low-density lipoprotein (LDL)–cholesterol concentrations[6,17].

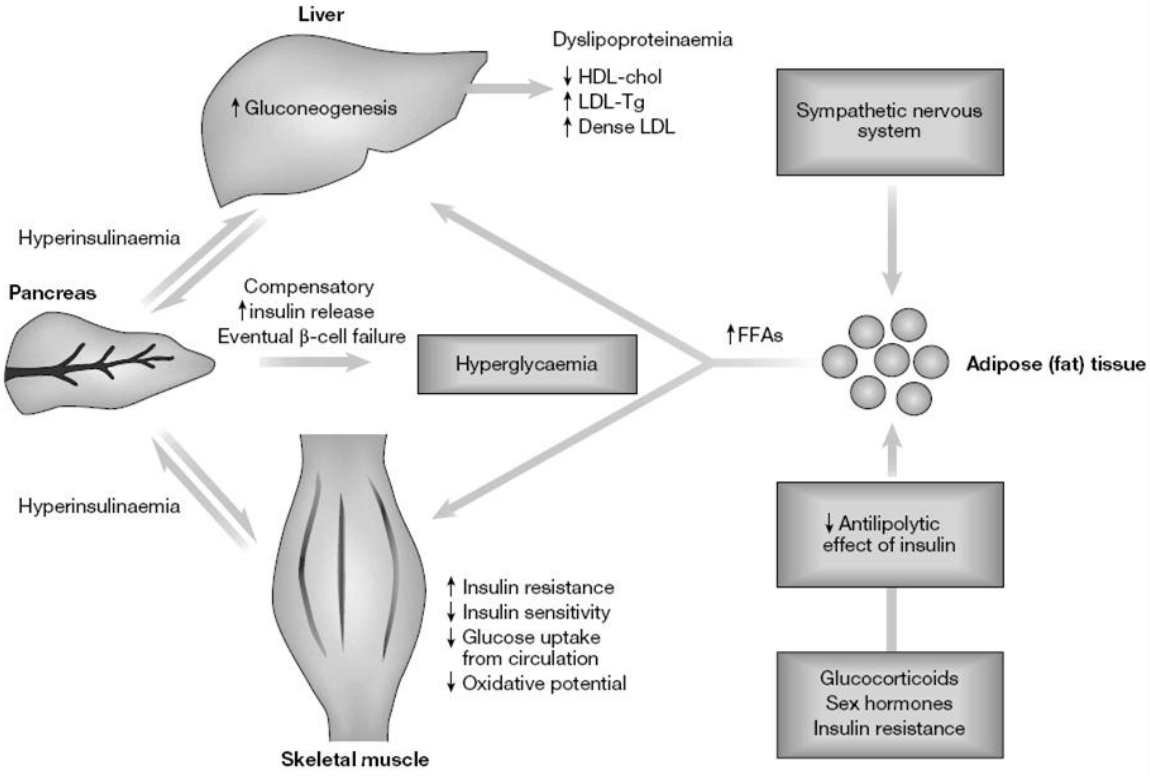


**Figure 2. Schematic illustration of the effects of increasing amounts of WAT on whole-body sensitivity to the actions of insulin and glucose.** (Figure adapted from[6].)

## 3.1.2 Obesity-induced hypertension

Obesity, especially visceral obesity, is strongly associated with arterial hypertension[18-19]. Since expanded lean tissue mass as well as oxidative demands of the metabolically active adipose tissue increases in obesity, total oxygen consumption is enhanced. Therefore, the total blood volume in obesity is increased in proportion to body weight. The central mechanism to increase the blood volume in obesity is enhanced sodium and water retention. Reasons for these changes in sodium handling might be a hyperinsulinaemia- and hyperleptinaemia-induced sympathetic nervous system activation, as well as activation of the renin-angiotensin-aldosterone-system (RAAS)[20]. Another important reason for the impaired pressure natriuresis in obesity is physical compression of the kidney that leads to alterations of intrarenal forces[20]. The expanded blood volume raises the left ventricular preload of the heart and in turn, cardiac output. As blood pressure is a function of cardiac output and systemic vascular resistance, the increased blood volume in obesity ascends blood pressure[6,17].

### 3.1.3 Obesity-induced inflammation

The WAT is a heterogeneous organ. In addition to adipocytes that are its most abundant cells, the WAT contains pre-adipocytes, which are adipocytes that are not loaded with lipids, stromal vascular cells, leukocytes and importantly, macrophages. Besides their function in fat storage, WAT is known to be an endocrine organ that releases a wide range of humoral cytokines and signaling molecules, called "adipokines". The most important adipokines, mainly secreted by adipocytes, are: leptin, tumor necrosis factor α (TNF-α), adiponectine, interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), and components of the renin-angiotensin-system (RAS)[21-22].

#### 3.1.3.1 Leptin

The protein hormone leptin is one of the most important adipokines of the WAT. It is mainly produced by adipocytes and plays a key role in regulating energy intake by acting on the hypothalamus and inhibiting appetite[23]. Furthermore, leptin influences the development of inflammation by inhibiting macrophage-mediated responses[24].

#### 3.1.3.2 TNF-α

The proinflammatory cytokine TNF-α is secreted by adipocytes[25], stromovascular cells[26] and macrophages[27]. It is found to be overexpressed in the adipose tissue of rodent models of obesity as well as in the adipose tissue of obese humans[28-29]. TNF-α is involved in the development of inflammation and insulin-resistance[25]. Adipocyte-derived TNF-α stimulates MCP-1 production by preadipocytes [30] and enhances the expression of adhesion molecules as vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) in endothelial cells[31]. These effects of TNF-α mediate the recruitment and adhesion of monocytes into the WAT[31].

#### 3.1.3.3 Adiponectin

The protein hormone adiponectin is mainly secreted by adipocytes and exhibits antiatherogenic and insulin-sensitizing properties[32]. In response to TNF-α induced inflammation in aortic endothelial cells, adiponectin enhances the expression of adhesion molecules as ICAM-1 and VCAM-1[33]. Low adiponectin levels are found in coronary artery disease, essential hypertension and diabetes mellitus[34].

#### 3.1.3.4 MCP-1

MCP-1 is a chemoattractant protein, that can bind to endothelial cells and recruits immune cells to the site of inflammation. MCP-1 is mainly produced by macrophages and adipocytes[35-37]. Its secretion is increased in the adipose tissue in obesity[38] and mediates visceral fat-induced atherosclerosis in mice[39].

#### 3.1.3.5 IL-6

The proinflammatory cytokine IL-6 is produced by immune cells and the adipose tissue[40]. It stimulates the synthesis of the acute phase response protein C-reactive protein (CRP) in the liver that in turn, activates the complement system[41] and has been associated with the development of type 2 diabetes mellitus[42].

#### 3.1.3.6 PAI-1

The serin protease inhibitor PAI-1 is a procoagulative agent since it is the principal inhibitor of fibrinolyes. PAI-1 secretion by adipocytes is increased in obesity[43] and plays an important role in thrombosis[44] as well as in tissue remodeling in atherosclerotic lesions in coronary artery disease[45].

#### 3.1.3.7 The renin-angiotensin-system

Adipocytes express all components of the RAS that are required for Angiotensin II (AngII) production and signaling: angiotensinogen (Ang), angiotensin-converting enzyme (ACE), renin and AngII receptors[46-47]. Indeed it has been shown that AngII is produced in the adipocytes and that the adipocytes themselves are sensitive to AngII[48-49]. It has still to be clarified if the AngII secreted by adipose tissue plays an important systemic role in the development of vascular inflammation and atherogenesis [50].

The adipokines produced by the WAT are released in the circulation and may influence other organs. Obesity is accompanied by an abnormal production of many adipokines, leading to an imbalance of complex interactions, and consequently to metabolic problems (Figure 3)[51]. Even a moderate weight reduction decreases circulating levels of proinflammatory adipokines as TNF-α and IL-6[52].

Since there are difference in the production of adipokines between visceral and peripheral fat tissue, the site of fat accumulation seems plays an pivotal role in the development of obesity-induced metabolic changes[53]. For example, the visceral fat tissue shows higher activity in secretion of adipokines as adiponectin, PAI-1 and Ang[26].

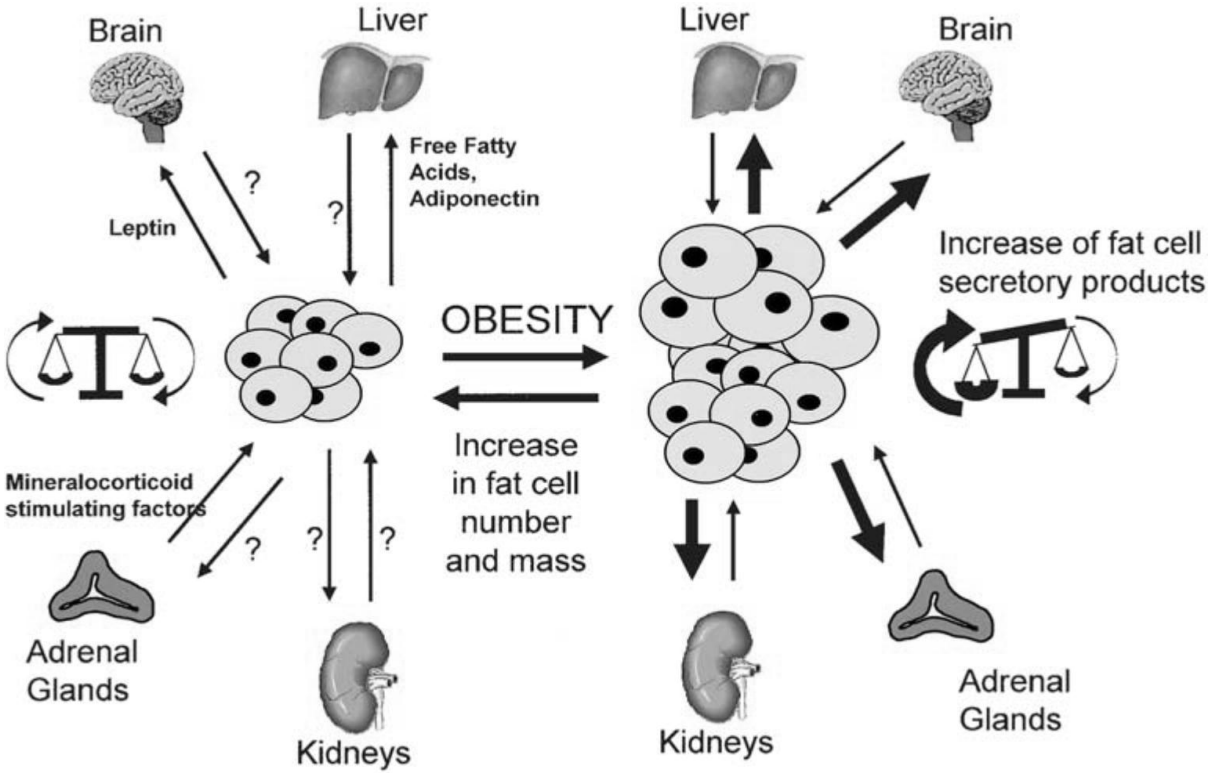


**Figure 3. Adipose tissue is a highly active endocrine organ that is involved in the body's metabolism.** Adipokines, secreted by the WAT, influence various organs in the body. Since obesity is associated with increased production of many adipokines, the homeostasis within the organism is disturbed. These pathological changes induce metabolic disorders. (Figure taken from[51]).

Besides dysregulated production of adipokines, the WAT itself is characterized by enhanced inflammation with increased infiltration of macrophages and T-cells in obesity[30,54]. It is suggested, that enhanced oxidative stress resulting from an increasingly lipolytic environment in obesity, induces physical damage to the endothelium in the WAT (Figure 4). The damaged endothelial cells produce MCP-1 as well as TNF-α and recruit macrophages to WAT, in the early state of obesity. In turn, the accumulating adipocytes secrete MCP-1, leptin and TNF-α and decrease the production of adiponectin; thereby contributing to macrophage accumulation[55]. This results in a viscous cycle of macrophage recruitment, production of inflammatory cytokine and impaired adipocyte function[55].

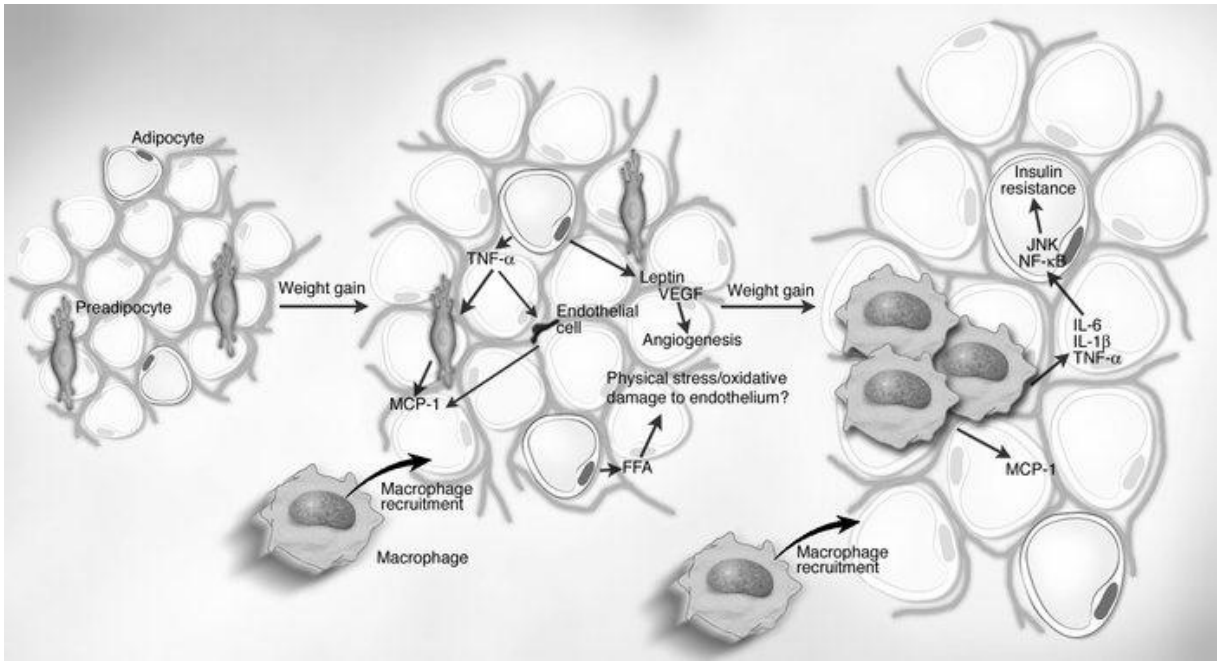


**Figure 4. Obese adipose tissue is characterized by inflammation and progressive accumulation of macrophages.** (Figure taken from [54].)

## 3.1.4 Obesity-induced oxidative stress

### 3.1.4.1 Reactive oxygen species (ROS)

Enhanced metabolic stress influences signaling pathways in the cell and in turn alters expression of specific genes. One form of such stress is the increased production of ROS. Oxidative stress plays a critical role in the development of various diseases and has been shown to be increased in obesity[56]. It is mainly caused by a dysbalance between the activity of endogenous pro-oxidative enzymes such as the NADPH oxidase, mitochondrial respiratory chain or xanthine oxidase and anti-oxidative enzymes as the superoxide dismutases (SOD), catalase, glutathione peroxidase, and paraoxonase.

Under physiological conditions, production and degradation of ROS is balanced and therefore, the redox state of the cell is well equilibrated. They can act as second messenger and influence signaling cascades, that regulate gene expression and posttranslational modification[57-58]. Increased release of ROS or deficiencies in the antioxidant systems elevate intracellular free radical concentration, which may initiate lipid peroxidation and induce pro-inflammatory redox-sensitive pathways. Two important mediators of transcription of pro-inflammatory genes are the redox sensitive transcription factors nuclear factor-kB (NF-kB) and activator protein-1 (AP-1)[59]. These transcription factors may induce expression of proinflammatory genes, including cytokines, cell adhesion, matrix metalloproteinases and inducible nitric oxide synthase (NOS)[60-61]. In turn, increased oxidative stress is central to various cardiovascular diseases such as atherosclerosis, congestive heart failure and hypertension[62].

ROS can be divided in 2 different classes: oxygen radical and non-oxygen radicals. The most important oxygen radicals are the superoxidanion ($O_{2^-}$) and the hydroxyradical (OH•). Since it is a precursor of several other radicals, the superoxidanion has central position within the ROS. Its most important interaction is the reaction with nitric oxide (NO), leading to a decrease in NO bioavailability in the vasculature[63] and to the generation of the harmful peroxynitrite ($ONOO^-$)[64]. Non-radical ROS include hydrogenperoxide ($H_2O_2$) and hypochloride (HOCl), both of which exert harmful effects by favoring free radical production

### 3.1.4.2 NADPH oxidases

One of the most prominent source of ROS in adipocytes and the vascular tissue is the NADPH oxidase[65-68]. This enzyme system catalyzes the transfer of a single electron from cytosolic NADPH to molecular oxygen leading to the generation of the superoxide radical. The NADPH oxidase complex comprises several subunits. The main component is the heterodimeric cytochrom $b_{558}$ that consists of the membrane-bound subunits p22phox and gp91phox. Its prosthetics groups FAD and Heme, that are involved in the electron transition from NADPH to the molecular oxygen, are bound by these two subunits[69] (Figure 4). In its inactive state, the

NADPH oxidase subunits p47phox, p67phox, p40phox and the GTPase Rac are located in the cytoplasm. Stimulation by phorbol ester or bacterial toxins induces a proteinkinase C-dependent phosphorylation of p47phox and activation of the Rho GTPase Rac. This in turn induces an activation and translocation of the cytosolic subunits to the plasma membrane[70]. The association with the subunits p22phox and gp91phox initiates superoxide generation[71]. The reducing substrate NADPH binds on the cytosolic side to the gp91phox subunits and is oxidized. The electrons are transferred to FAD, the first and second Heme group and are subsequently accepted by molecular oxygen that is thereby reduced to the superoxidanion (Figure 5)[72].

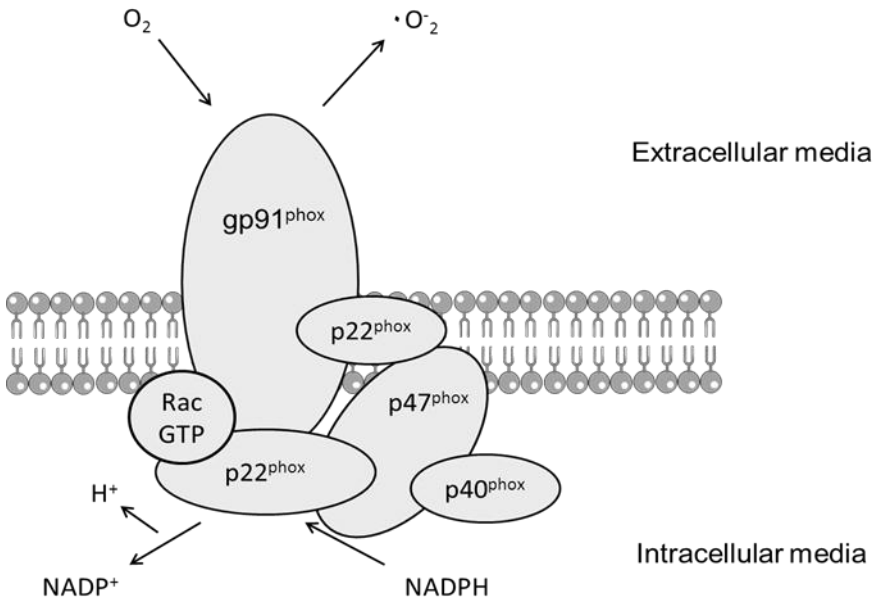


**Figure 5. The non-phagocytotic NADPH oxidase complex.** The NOX subunit is bound to p22phox in the plasma membrane. Upon activation, the cytosolic subunits translocate to the plasma membrane and allow the generation of superoxide.

The catalytic subunit of the NADPH-oxidase gp91phox is a member of a protein family, that show high similarity to the neutrophilic NADPH oxidase and are called non-phagocytotic NADPH oxidases (NOX)[72]. NOX2 (also called gp91phox) is mainly expressed in endothelial cells and phagocytes, whereas NOX1, NOX4 and NOX5 are localized in endothelial cells, vascular smooth muscle cells and other cell types[73-74]. Adipocytes express exclusively NOX4[65]. NOX3 is primarily found in embryonic tissue[75].

### 3.1.4.3 Antioxidant systems

To protect from oxidative damage by ROS, reducing antioxidant systems as superoxide dismutase (SOD) or catalase regulate the ROS content in the cell[72]. SODs are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. They are an important antioxidant defense in nearly all cells exposed to oxygen. Several common forms of SOD exist: cofactored with copper and zinc (SOD-1 and SOD-3) or manganese (SOD-2). Whereas SOD-1 and SOD-2 are located intracellular, the SOD-3 is located extracellular between the endothelium and the vascular smooth muscle cells (VSMCs). Catalase is another intracellular antioxidant system that catalyzes the decomposition of hydrogen peroxide to water and oxygen.

### 3.1.4.4 ROS action in WAT

Indeed, a direct correlation between obesity and oxidative stress, assessed by measuring $F_2$-isoprostanes, has been demonstrated in humans[56]. Isoprostanes are prostaglandin-like products that are formed *in vivo* via a non-enzymatic mechanism and involve the free radical-mediated peroxidation of arachidonic acid. $F_2$-isoprostanes measured in the plasma and in the urine are biomarkers for lipid oxidation and therefore for oxidative stress[76]. Besides obesity, $F_2$-isoprostanes are increased in diabetes[77], hypercholesterolemia[78] and end-stage renal disease[79].

Furukawa and colleagues[65] demonstrated that ROS production is increased in the entire adipose tissue of mice and cultured adipocytes. This increased oxidative stress is accompanied by enhanced expression of the NADPH oxidase as well as attenuated expression of SOD-3 and catalase in the WAT[65]. This is in line with another publication showing attenuated SOD-3 expression in obesity, thereby contributing to enhanced oxidative stress[80-81]. The adipose tissue derived ROS in obesity dysregulates the expression of adipokines as adiponectin, PAI-1, IL-6 and MCP-1. Inhibition of the NADPH oxidase resulted in attenuated ROS production, normalized adipokines production and ameliorated diabetes and hyperlipidemia[65].

Taken together, the increased oxidative stress in obesity might enhance proinflammatory changes in remote tissues in two ways: directly, by activating redox-sensitive nuclear transcription factors as AP-1 and NF-κB and indirectly, by dysregulating adipokines expression in the WAT that in turn may affect target tissues. The proinflammatory changes in target tissues might contribute to development of insulin resistance, diabetes and atherosclerosis (Figure 6). Thus, the proinflammatory and pro-oxidant effects in obesity may link obesity and cardiovascular disease.

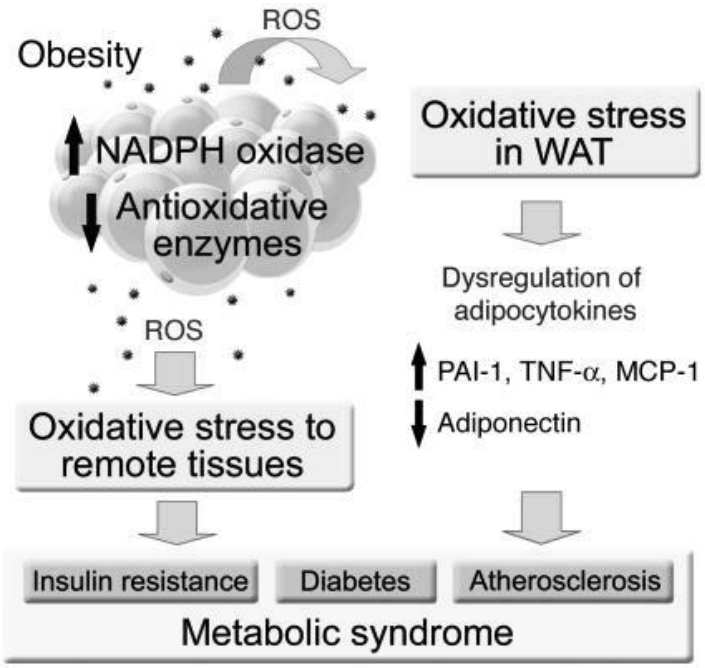


**Figure 6. Increased ROS production in accumulated fat contributes to metabolic syndrome**. (Figure taken from[65]).

# 3.2 Blood vessel wall

## 3.2.1 Structure of the blood vessel

The blood vessels are an important part of the cardiovascular system as they distribute the blood, oxygen and nutrients to the organs, and remove the waste products from tissues throughout the body. All blood vessels, except capillaries, have the same basic anatomical structure (Figure 7).

The tunica intima is the inner layer of a vessel, consisting of one coat of endothelial cells and is in direct contact to the flowing blood in the vessel. The endothelium separates the blood from the deeper layers (elastic interna and the underlying vascular smooth muscle).

The tunica media consists, depending on the type of vessel, of a more or less pronounced layer of VSMCs that is lined on both sides by an elastic lamella of connective tissue. The tunica media is responsible for the regulation of the vascular tonus and is a target of hormonal and neuronal signals[82].

The outer layer, the tunica externa (adventitia) consists mainly of fibroblasts and connective tissue that anchors the vessel in the surrounding tissue. In addition, nerves and nutrimental blood vessels can be located in tunica externa [83].

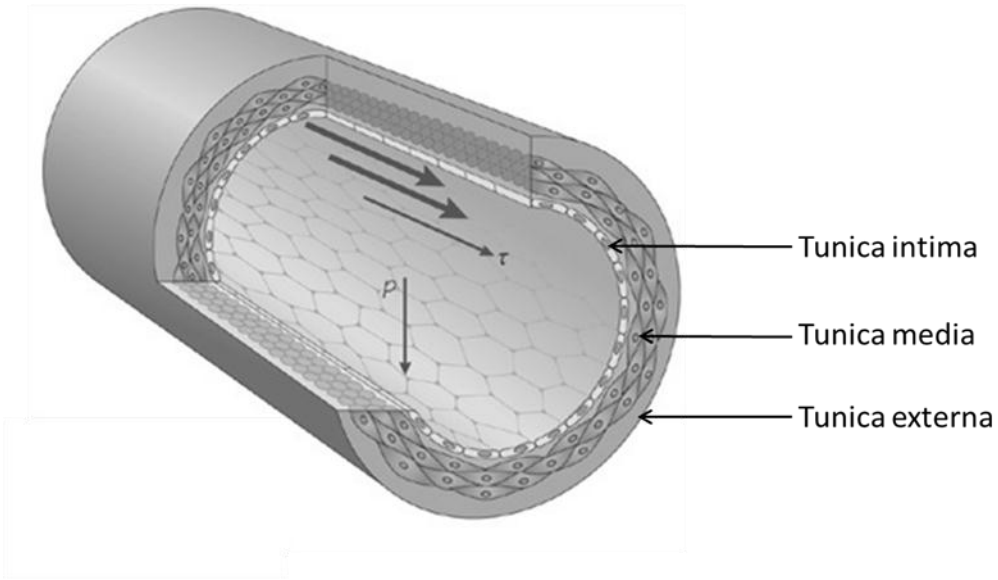


**Figure 7. Structure of a muscular artery.** The three structural components of the blood vessel, the intima, media and adventitia are indicated. Mechanical forces as pressure (p) and shear stress (τ) act luminal on the vessel wall. (Figure adapted from[84].)

### 3.2.2 The endothelium

The endothelium is the largest organ in the body. It forms the border between the circulating blood in the vessel lumen and the rest of the vessel wall. The endothelium senses mechanical stimuli such as pressure, shear stress (Figure 7) and hormonal stimuli, such as vasoactive substances. The various functions of the endothelium comprise the regulation of the vessel tone, control of endothelial permeability and inhibition of thrombocyte aggregation, thrombocyte and leukocyte adhesion on the vessel surface and smooth muscle cell growth[85-86]. These effects are controlled by various growth factors and vasoactive mediators that influence the physical and biochemical properties of the vessel.

The vascular tone of the vessel is determined by a well-orchestrated release of vasodilators and vasoconstrictors. The most important endothelium-derived relaxing factors (EDRF) are NO and prostacyclin ($PGI_2$). Endothelium-derived contracting factors (EDCF) comprise endothelin-1, ROS, angiotensin-II, thromboxane $A_2$, prostaglandin $F_{2\alpha}$ and under pathophysiological conditions, prostacyclin[87-89].

Since it has been discovered, that the neurotransmitter acetylcholine requires intact endothelial cells to induce arterial vasodilatation, the importance of the endothelium for vascular homeostasis has been increasingly recognized[90] (Figure 8). Under physiological conditions the neurotransmitter acetylcholine binds to specific G protein coupled receptors on endothelial cells and the downstream signaling pathway leads to phosphorylation and activation of the endothelial nitric oxide synthase (eNOS)[91]. The eNOS uses L-arginine as substrate to generate L-citrulline and NO in a NADPH-and $O_2$-dependent manner. NO produced in the endothelial cells diffuses into the underlying VSMCs and stimulates the NO-sensitive guanylylcyclase (GC); the most crucial receptor for NO[92-93]. The GC induces cyclic guanosin monophosphate (cGMP) production by activating soluble GC. In turn, cGMP mediates than most of its intracellular actions by activating cGMP-dependent protein kinases (PKG), that influences the vascular tone of the VSMC[92]. Besides from eNOS two other isoforms have been identified so far : the inducible NO synthase (iNOS) and the neuronal NO synthase (nNOS)[91,94]. Nevertheless, eNOS seems to play the most prominent role in inducing endothelium-dependent vasodilation in the healthy and diseased vessel[95-96]. NO is the major endothelium-derived relaxing factor in the vascular system. In addition, NO exerts vascular protective effects such as prevention of monocyte adhesion to the endothelial layer, reduction of smooth muscle cell proliferation, and inhibition of platelet activation and aggregation[97].

Another important EDRF is $PGI_2$, the principal metabolite of arachidonic acid in endothelial cells[98-99]. Acetylcholine induces the generation of $PGI_2$, from arachidonic acid in several enzymatic steps, including the cyclooxygenase (COX) and prostaglandine synthase. $PGI_2$ is the preferential ligand for the prostacyclin receptor (IP) on smooth muscle cells that activates AC and thus elevates intracellular cAMP. This induces vasodilation[100].

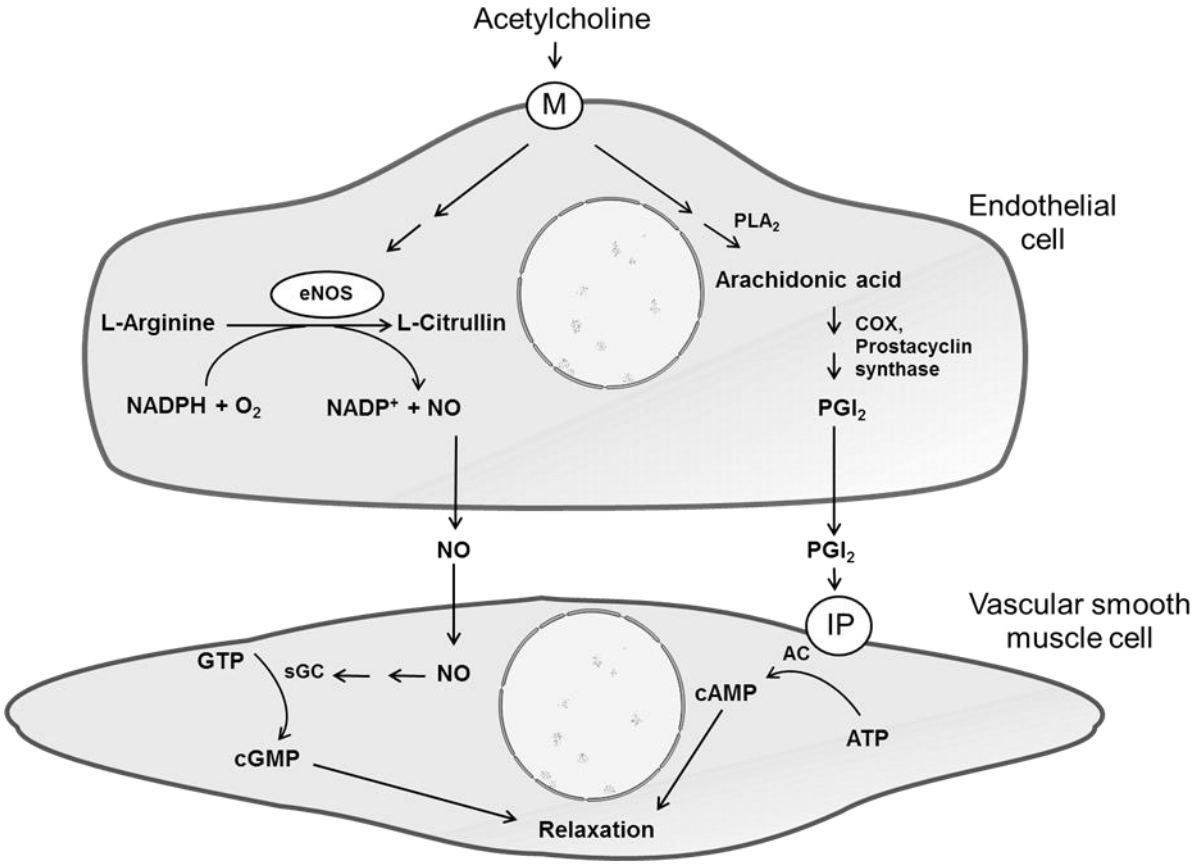


**Figure 8. Mechanism of endothelium-dependent relaxation by acetylcholine.** M muscarinic receptor, *eNOS* endothelial NO synthase, *NO* nitric oxide, *sGC* soluble guanylatecyclase, *cGMP* cyclic guanosin monophosphate, *$PLA_2$* phospholipase 2, *COX* cyclooxygenase, *$PGI_2$* prostacycline, *IP* prostacyclin receptor. (Figure adapted from[89,101].)

## 3.2.3 Endothelial dysfunction

An intact endothelium is required for healthy physiological vascular function. The protective function of the endothelium can be impaired by various factors as infections, systemic or local inflammation, hypertension, pharmaceuticals and metabolic disorders. Loss of proper endothelial function may lead to chronic endothelial dysfunction that is an important early step in atherogenesis[102]. It appears years before any obvious clinical, pathological changes of the vessel wall are observed.

Endothelial dysfunction has been associated with diseases contributing to the development of cardiovascular diseases as hypertension[103], obesity and metabolic syndrome[104-105], type 1 and type 2 diabetes[106-107] as well as with cardiovascular disease itself like, coronary artery disease[108], congestive heart failure[109], chronic renal failure[110]. Endothelial dysfunction is characterized by a shift of actions of the endothelium towards reduced vasodilation or even vasoconstriction, a proinflammatory state, and prothrombotic properties. Impaired endothelium-dependent vasodilatation may arise from several mechanisms including: decreased production of one of the EDRFs, enhanced inactivation or impaired diffusion of these EDRFs to the underlying smooth muscle cells and enhanced generation of EDCFs.

### 3.2.3.1 Decreased production of EDRF NO

NO is one of the most important vasodilatory substances released by the endothelium. Reduced NO levels are often associated with endothelial dysfunction[97]. These reduced amounts of NO might be due to a decreased eNOS activity or a decreased bioviability of NO. Increased oxidative stress, which is often observed in patients with hypertension, hypercholesterolemia or diabetes, impairs NO production and chemically quenches NO by formation of toxic peroxynitrite[97,111]. In addition, superoxide enhances the degradation of 4-tetrahdrobiopterin (BH4), a cofactor of eNOS, leading to 'uncoupled' eNOS. This means that the oxygen reduction is uncoupled from the NO synthesis, thereby yielding a dysfunctional superoxide generating enzyme that produces ROS and therefore enhance vascular oxidative stress[112-113].

### 3.2.3.2 Enhanced generation of EDCF

**ROS**

The NADPH oxidase is the most important source of ROS in endothelial cells and VSMCs in quiescence and under stimulation[67-68]. As already mentioned above, the NADPH oxidase-derived ROS reduces NO bioavailability. In addition to that, ROS induce vasoconstriction directly and enhance redox-sensitive transcription factors as NFκB that can initiate vascular inflammation[114-115]. ROS also induce lipid peroxidation, endothelial cell apoptosis, matrix metalloproteinase (MMP) expression and proliferation of vascular smooth muscle cells. All of these effects promote endothelial inflammation and dysfunction[116]. Indeed, in animal models of

diabetes, increased oxidative stress enhances the development of endothelial dysfunction[117-118]. In hypertension and chronic renal failure, it has been shown that treatment with antioxidants modulate enzyme systems that generate free radicals, namely NADPH oxidase and SOD[119-120] in a beneficial way and attenuate endothelial dysfunction by increasing eNOS activity[121]. Beneficial effects of antioxidant treatment has been also observed in endothelial dysfunction associated with obesity[122].

**Angiotensin II**

AngII contributes to the development of hypertension and endothelial dysfunction by inducing vasoconstriction and by enhancing NADPH oxidase generated ROS in aortic endothelial cells[123-127].

**Prostanoids**

Endothelial dysfunction can be due to the enhanced release of endothelium-derived contracting factors that counteract via prostanoids receptors (TP) the vasodilatory action of NO. These endothelium-derived contractions involve the endothelial COX-1 and COX-2 activation and release of various prostanoids that activate the TP on the underlying vascular smooth muscle cells. This in turn, results in vasoconstriction of the vessel (Figure 9)[89,128].

Prostanoids are a subclass of eicosanoids consisting of prostaglandins, thromboxanes and prostacyclins. They are enzymatically derived from fatty acids and exert physiological effects, such as relaxation and contraction of smooth muscle tissue[89]. Arachidonic acid, the most common precursor of prostaglandins, is generally produced from phospholipids by phospholipase $A_2$ ($PLA_2$) and can be metabolized either via the lipoxygenase pathway to form leukotriens or via the cyclooxygenase pathway to form prostaglandins. Prostaglandins are produced following sequential oxidation by COX and terminal prostaglandin synthases[129].

The cyclic endoperoxide Prostaglandin $H_2$ ($PGH_2$), produced by COX, can be metabolized into prostaglandin $D_2$ ($PGD_2$) by the PDG synthase, into prostaglandin $E_2$ ($PGE_2$) by the PGE synthase, into prostacyclin ($PGI_2$) by the prostacyclin synthase and into thromboxane ($TXA_2$) by the thomboxane synthase, respectively.

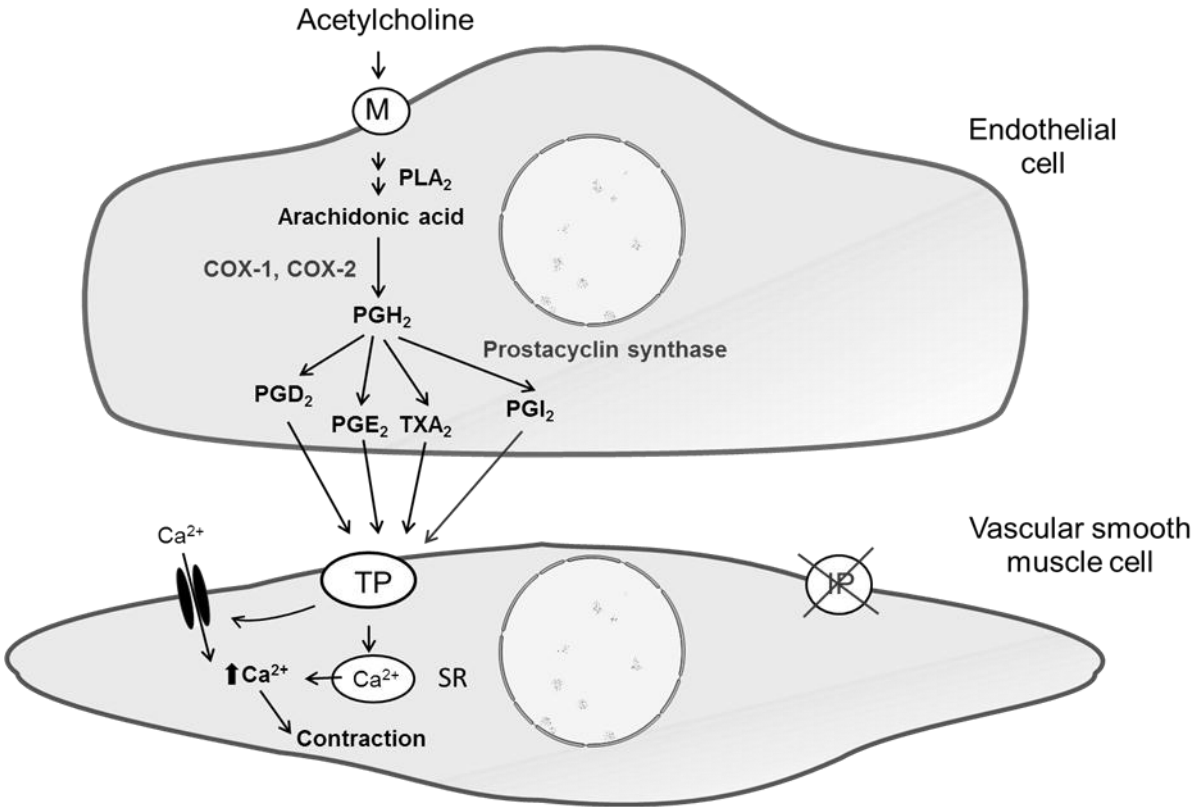


**Figure 9. Mechanism of endothelium-dependent contractions by prostanoids.** The red color indicates pathophysiological changes that can contribute to endothelium-dependent contractions. M muscarinic receptor, $PLA_2$ phospholipase 2, COX-1 cyclooxygenase-1, *COX-2* cyclooxygenase-2, *$PGH_2$* prostaglandin $H_2$, *$PGD_2$* prostaglandin $D_2$, *$PGE_2$* prostaglandin $E_2$, *$TXA_2$* thromboxane, *$PGI_2$* prostacycline, *TP* prostanoid receptor, *IP* prostacyclin receptor, *SR* sarcoplasmatisches reticulum. (Figure adapted from[89,101].)

COX-1 is expressed in most tissues constitutively, while COX-2 is induced mainly at sites of inflammation[130]. Enhanced endothelial expression of COX-2 enhances endothelial contraction in aged and hypertensive rodents[131-132]. Inhibition of COX by indometacine abolishes endothelial dysfunction in diabetic rats whereas COX-1 derived products seem to play the major role in this case[133].

Prostaglandins bind seven-transmembrane, G-protein-coupled receptors, most importantly to mention because they are expressed on endothelial cells are the TP and the IP. $TXA_2$, $PGH_2$ and other prostaglandins can activate the TP[134] and promote vasoconstriction by increasing intracellular calcium. Furthermore, they enhance expression of adhesion molecules and platelet aggregation[135].

As already described above, $PGI_2$ acts as a vasodilator under physiological conditions by binding to the IP receptor[98-99]. Paradoxically, $PGI_2$ has been show to be a major constricting factor in hypertension. Because of specific dysfunction of the IP in the VSMC, $PGI_2$ binds to the TP and induces TP-dependent contractions[136-137].

#### 3.2.3.3 Effects of adipokines on endothelial dysfunction

As endothelial dysfunction is one of the early stages of atherosclerosis, it is reasonable to appreciate that adipokines, secreted by the WAT in obesity, might have a direct impact on endothelial cells and contribute to the development of endothelial dysfunction.

It has been shown that increased leptin levels under caloric restriction improve acetylcholine-induced NO-dependent vasorelaxation in the rat aorta[138]. In addition, endothelium-dependent relaxation is deteriorated in subjects with low plasma adiponectin levels [139]. In the liver, the cytokine IL-6 stimulates the synthesis of acute-phase response proteins such as CRP, which induce endothelial dysfunction[140]. Similarly, TNF-α induces enhanced endothelial permeability by activating NADPH oxidase[141] and inhibits transcriptional and post-transcriptional eNOS gene expression[142]. Both of these effects are known to enhance endothelial dysfunction. Besides from the strongly altered secretion of adipokines, obesity is associated with increased concentrations of FFA in the plasma. Increased plasma levels of the FFA oleic acid inhibits eNOS activity in aortic endothelial cells *in vitro*[143] and impairs endothelial function in healthy subjects *in vivo*[144]. Thus, adipokines and FFA contribute directly to the development of endothelial dysfunction in obesity.

### 3.2.4 Pathophysiological effects of endothelial dysfunction

Apart from an impaired vasodilation, endothelial dysfunction is characterized by a pronounced proinflammatory state. Low NO bioavailability and enhanced oxidative stress in endothelial cells results in the activation of the redox-sensitive NF-κB signaling pathway. Activated NF-κB migrates into the nucleus and induces the expression of numerous genes, including many pro-inflammatory genes such as E-Selectin, ICAM-1, and VCAM-1[61,145]. These endothelial cells are considered 'activated'. In contrast, under physiological conditions aortic endothelial cells do not express these adhesion molecules, except for low constitutive levels of ICAM-1[146].

Presentation of these molecules play a role in the initiation of inflammatory process since they attract and activate blood monocytes, which then transmigrate into the arterial intima. NO inhibits leukocyte adhesion[147]. This transmigration of monocytes is stimulated by chemokines, such as MCP-1, MCP-4, regulated on activation normal T cell expressed and secreted (RANTES), and IL-8, which bind to G protein coupled-receptors and are expressed by various vascular cells[148].

Adhesion of mononuclear phagocytes to the activated endothelial layer starts early in atherogenesis[149], suggesting that endothelial activation is an early inflammatory step of the disease. Subendothelial macrophages transform into lipid-laden foam cells, they scavenge oxidized LDL through receptors such as LOX-1[150]. As the atherosclerotic plaque grows, growth factors that are secreted by macrophages in the plaque, stimulate vascular smooth muscle cell proliferation and interstitial collagen synthesis[151]. Decreased NO and increased oxidative stress active MMP-2 and MMP-9 that may weaken the fibrous cap[151-152] and eventually lead to the rupture of the thin fibrous cap, thrombus formation with complete coronary occlusion, and myocardial infarction. All these proinflammatory events, participating in atherogenesis, underline the concept that atherosclerosis is a chronic inflammatory disease[152].

## 3.3 Aldosterone and the renin-angiotensin system

The RAAS is a hormone system that regulates blood pressure and volume homeostasis. Low blood pressure induces the juxtaglomerular cells in the kidneys to secrete renin. Renin stimulates the production of $\alpha_2$-globulin Ang in the liver and cleaves Ang into the decapeptide AngI. In turn, AngI is cleaved by the endothelial enzyme ACE into the octapeptide Ang II (Figure 10). By binding to its receptor, $AT_1$ receptor (type I ANG II receptor), Ang II can act directly as a potent vasoconstrictor. In addition, Ang II induces renal sodium reabsorption, stimulates thirst and AVP secretion in the central nervous system (CNS). Furthermore, it induces a signaling pathway in glomerulosa cells of the adrenal gland that results in increased aldosterone synthesis and release. Ang II also binds to $AT_2$ receptors (type II ANG II receptor) inducing a counter regulatory vasodilation. The final endocrine hormone of the RAAS is the mineralocorticoid aldosterone that is the prime regulator of salt balance and extracellular volume in the body[17,153].

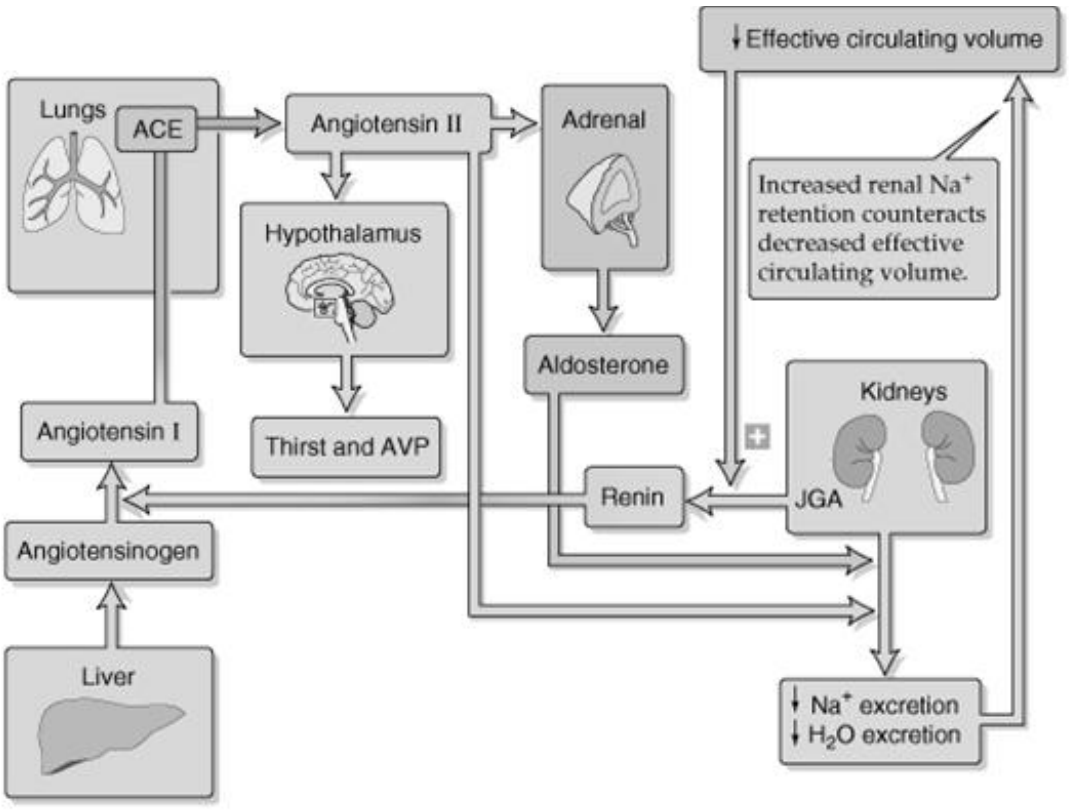


**Figure 10. The renin-angiotensin-aldosterone axis.** *JGA* juxtaglomerular apperatus.(Figure taken from the Textbook Boron & Boulpaep[17])

### 3.3.1 Aldosterone

Aldosterone regulates the blood volume by increasing $Na^+$ absorption in the distal kidney tubules and collecting ducts in exchange for $K^+$. This causes enhanced retention of water, which elevates the extracellular fluid volume and in turn, raises arterial pressure. Moreover, aldosterone enhances blood pressure by inducing vasoconstriction in the vasculature and influences the central nervous system directly[154]. Since aldosterone plays a key role in the maintance of blood pressure, its release is tightly regulated by the RAS, extracellular $K^+$ and ACTH (adrenocorticotropic hormone). Furthermore, plasma acidosis and stimulation of stretch receptors, located in the atria of the heart, can enhance aldosterone secretion[155-156]. Over a 24h period, aldosterone displays an episodic secretory pattern, whereas mean plasma levels, pulse frequency and amplitude increase during the night and the early morning hours, in humans[157].

Aldosterone belongs to the class of steroid hormones and is synthesized from cholesterol in a series of five steps (Figure 11). The last of these steps is the conversion of corticosterone to aldosterone, catalyzed by the aldosterone synthase (cytochrome P-450 CYP11B2).

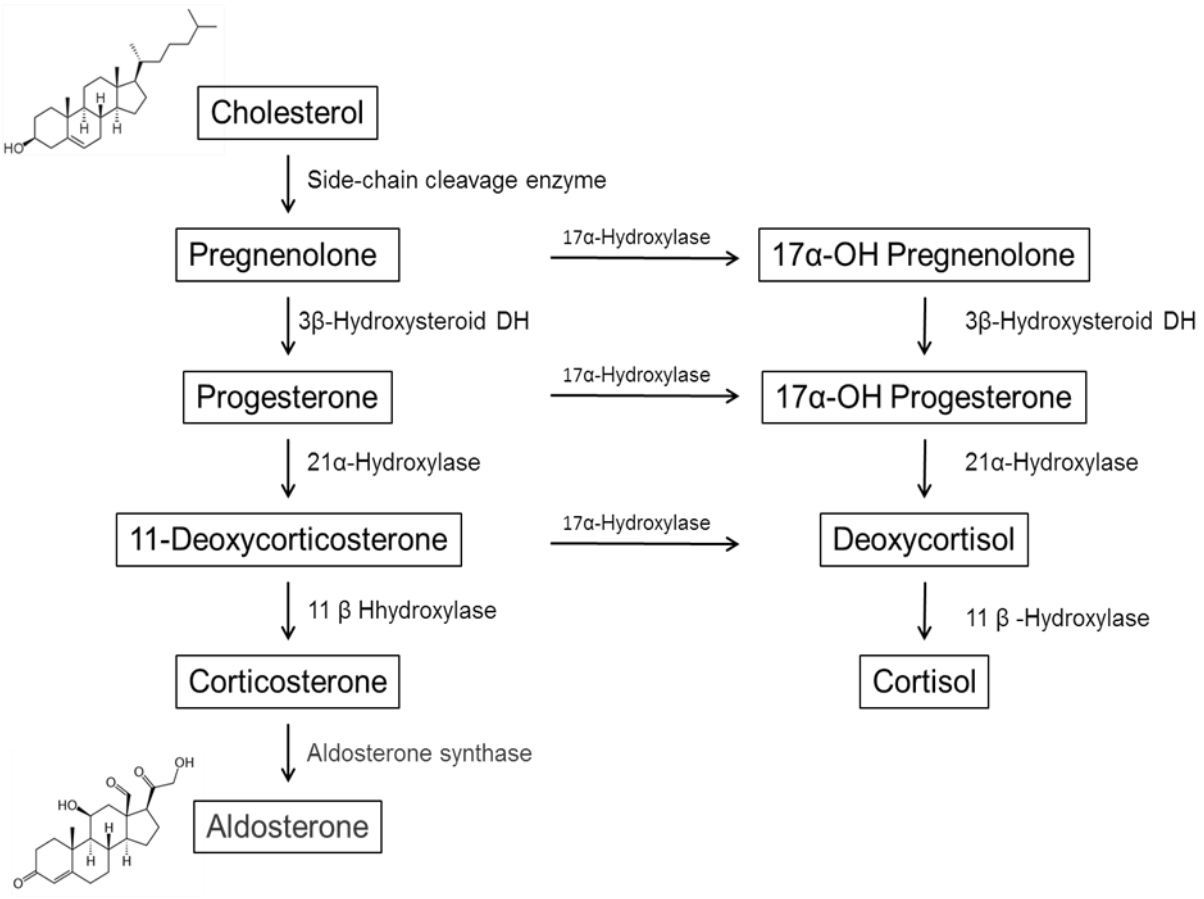


**Figure 11. Aldosterone synthesis form cholesterol.** (Figure adapted from[17].)

The aldosterone synthase, primarily expressed in the cortical adrenal gland of the kidney, controls the aldosterone secretion in the circulation. Even more, aldosterone can be produced in extrarenal tissues such as the heart[158] and the vascular system[159]. At the vascular level, aldosterone production has been demonstrated clearly in smooth muscle cells[160] whereas aldosterone production in vascular endothelial cells is still debated[66,161].

### 3.3.2 Mineralocorticoid receptor

Since aldosterone is a steroid hormone, it can freely diffuse through the plasma lipid membrane of the cells. In the cytoplasm, the hormone can bind to two different receptors; its low-affinity receptor, the glucocorticoid receptor (GR) and more importantly, its high-affinity receptor the mineralocorticoid receptor (MR). The MR, is also known as aldosterone receptor, or nuclear receptor subfamily 3, group C, member 2, (NR3C2) belongs to the steroid receptor subfamily, together with the glucocorticoid, progesterone and androgen receptors.

Steroid receptors display a modular structure comprised of three major functional domains (Figure 12). A N-terminal domain (NTD), followed by a central DNA-binding domain (DBD), and a hinge region that links them to the C-terminal ligand-binding domain (LBD). The human MR gene consists of 10 exons, including 2 untranslated first exons (1α and 1β). The translation start codon AUG starts at exon 2, whereas the stop codon UGA is located in exon 9. Exon 2 encodes most of the NTD, exons 3 and 4 encode each of the two zinc fingers of the DBD. Exons 5 to exon 9 encode for the LBD and mediate numerous functions, including ligand binding and interaction with heat-shock proteins[162].

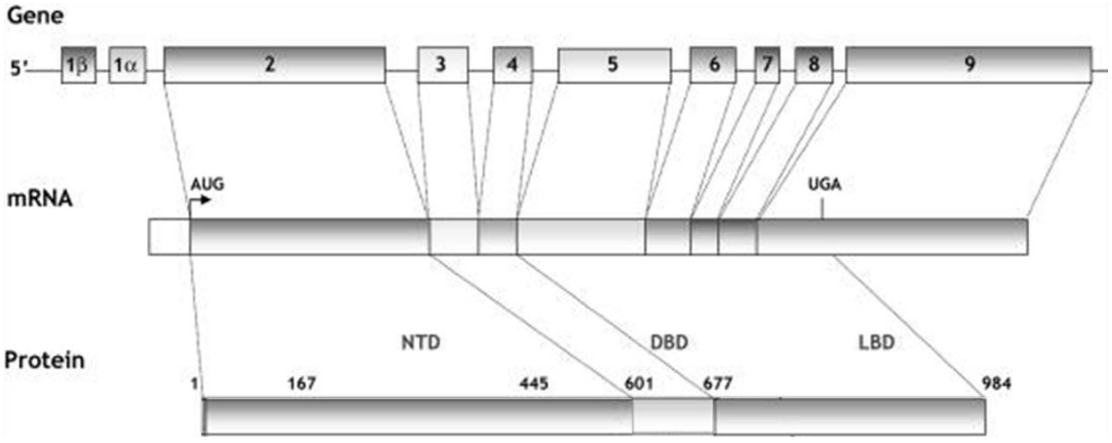


**Figure 12. Schematic representation of human MR structure.** *NTD* N-terminal domain, *DBD* DNA-binding domain, *LDB* ligand-binding domain. (Figure adapted from[162].)

The MR itself can be activated upon binding of aldosterone and cortisol[163]. Since cortisol has the same affinity to the MR as aldosterone and circulates at much higher concentrations in the plasma, only removal of the cortisol from aldosterone-targeted tissues enables aldosterone action. Indeed, various tissues express an enzyme called 11β-hydroxysteroid dehydrogenase-2 (11β-HSD2) that converts cortisol into its 11-keto analogs cortisone, which has lower affinity to the MR (Figure 13)[164-165]. As a result, locally within a target cell, the actual cortisol/aldosterone ratio is much smaller than the cortisol concentration seen in the plasma. Classical mineralocorticoid targets in the kidney, as the distal convoluted tubule and the initial cortical collecting duct, express high levels of 11β-HSD2[166]. Additionally, 11β-HSD2 protein has been detected in cardiac myocytes[167], vascular smooth muscle cells [168-169] and aortic endothelial cells[170-172], emphasizing their role as aldosterone target cells.

Tissues that do not express 11β-HSD2 are resistant to aldosterone-induced MR activation. Even more, cells can express the 11β-hydroxysteroid dehydrogenase-1 (11β-HSD1) that mediates reduction of cortisone to the active hormone cortisol and serve as tissue-specific amplifier of glucocorticoid action[173]. 11β-HSD1 is found in metabolic tissues as the liver, adipose tissue, and the CNS. In these tissues, cortisol activates both the MR and GR.

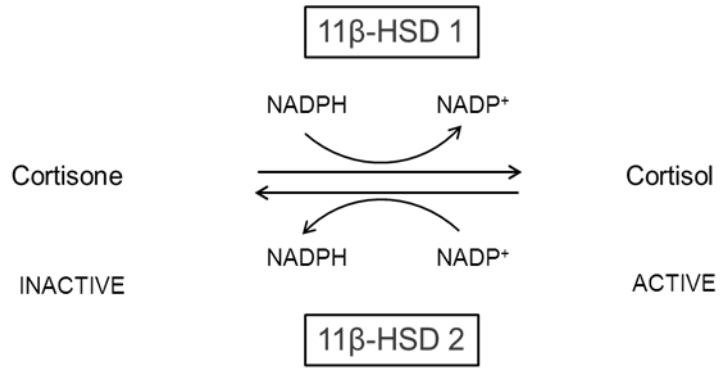


**Figure 13. Hydroxsteroid dehydrogenase action.** The tissue-specific enzyme 11β–HSD2 converts cortisol to MR-inactive cortisone in a NADPH-dependent manner. Vice versa, 11β–HSD1 converts the inactive cortisone into the active cortisol.

The major role of aldosterone is to regulate ion transport. Accordingly, the MR is expressed in epithelial tissues in which $Na^+$ transport is regulated such as the distal nephron of the kidney, the distal colon, the sweat glands as well as the salivary glands. Beyond its expression in these epithelial tissues, the MR is detected in the brain and pituitary[174-175] as well as in the cardiac and vascular system[170-171,176-177].

In an unliganded state, the MR is located in the cytoplasm and is complexed with the chaperones heat shock proteins 70, heat shock protein 90 and immunophilins[162,178]. Upon binding with aldosterone, the receptor-ligand complex dissociates from chaperons and translocates into the nucleus. In the nucleus, the MR directly binds to the DNA at its cognate binding site called steroid response element (SRE). The SRE is located in the regulatory region of target gene promoters and enhances gene expression. Furthermore, the activated MR can interfere with or induce transcription by protein-protein interactions with other factors that influence gene transcription[162].

In classical polarized epithelial tissues, aldosterone-activated MR induces the $Na^+$ absorption from the lumen by inducing the expression of amiloride-sensitive epithelial sodium channel (ENaC) at the apical membrane[179-180] and enhancing the activity of the $Na^+/K^+$-ATPase on the basolateral membrane. $K^+$ diffuses passively to the lumen through $K^+$ channels at the apical membrane. Along these lines, the MR alters the expression of serum-and glucocorticoid-induced kinase 1 (SGK1), neuronal precursor cell expressed developmentally downregulated 4 (nedd4), nedd4 isoform 2 (nedd4-2), K-Ras2A, and caspacin. All these mediators influence the expression of the ENaC and $Na^+/K^+$-ATPase in the cortical collecting ducts and distal tubules of the kidney, thereby modulating $Na^+$ reabsorption and $K^+$ secretion[166,181-182].

Additionally, MR activation by aldosterone induces rapid, non-genomic effects; they are insensitive to transcription inhibitors and comprise several second messenger systems: Various studies demonstrated that MR signaling enhances the extracellular-regulated kinase/mitogen-activated protein (ERK/MAP) kinase pathways, the $IP_3$ (inositol 1,4,5-trisphosphate)/$Ca2^+$ pathway as well as phosphorylation of c-SRC, JNK and NFκB[183-186]. Another non-genomic response of aldosterone involves an unidentified membrane receptor besides the MR[187]. It has been shown in MR knockout mice that aldosterone treatment enhances $Ca^{2+}$ and cAMP in fibroblasts and skin cells within minutes, independently of the MR[183,188]. In addition to the above-described actions, the activated MR is able to cross-activate other receptors, such as the epidermal growth factor and the $AT_1$[166].

### 3.3.3 Role of aldosterone in hypertension

As described in the previous sections, aldosterone plays an important role in regulating blood pressure. Various clinical studies described that hyperaldosteronism is a common cause of resistant hypertension[189]. Especially the pathophysiological overproduction of aldosterone by an adrenocortical adenoma (Conn`s syndrome) induces sustained hypertension[190]. Furthermore, it has been demonstrated in an animal model of spontaneously hypertensive rats, that cardiac and vascular aldosterone production contribute the development of hypertension [191-192].

For many years, inhibition of ACE and angiotensin receptor blockers (ARB) were used to lower aldosterone levels and treat its deleterious effects[193]. Surprisingly, aldosterone levels further increased, even if ACE inhibitors and ARBs were administered, the so-called "aldosterone-escape" phenomenom[193-195]. The reason for aldosterone-escape is still incompletely understood, but might be due to incomplete ACE inhibition or multiple pathways of aldosterone secretion[194]. Therefore, the direct blockade of the MR seems to be the best way to prevent the harmful effects of aldosterone excess.

Spironolactone, a well-known MR antagonist, was discovered in 1960, and has been proved to be very effective for treatment of resistant hypertension[196]. Due to its anti-androgenic properties, spironolactone shows unwanted side effects like gynaecomastia and impotence in males, and menstrual disturbances in females[197]. Therefore, a new more selective MR antagonist though with lower affinity, eplerenone, was developed and approved in 2002. MR blockade by eplerenone is beneficial in treating essential hypertension as a diuretic that improves blood pressure, left ventricular hypertrophy and proteinuria[198]. Eplerenone is marketed by Pfizer as Inspra®.

### 3.3.4 Role of aldosterone in obesity

Obesity is associated with hypertension. One of the central reasons is chronically enhanced sodium and water retention due to enhanced RAAS activation [20]. Indeed, diverse clinical studies showed a correlation between visceral obesity and increased plasma aldosterone levels[199-202]. Along this line, weight loss of obese humans is associated with attenuation in plasma aldosterone levels and arterial blood pressure[202-203].The importance of aldosterone in obesity-induced hypertension has been shown in a study with diet-induced obese dogs. MR antagonism clearly abolished obesity-induced sodium retention and hypertension[204]. Besides aldosterone, plasma Ang[205] and plasma ACE activity are increased in obesity[203]. In contrast, renin activity is not enhanced in obesity suggesting renin-independent aldosterone secretion in obese subjects[199,202].

Obesity is characterized by increased plasma fatty acid levels and oxidative stress. Therefore, the potential stimulatory function of oxidized fatty acids on aldosterone secretion was investigated. Indeed, an oxidized derivate of linoleic acid, 12,13-epoxy-9-keto-10(*trans*)-octadecenoicacid (EKODE), stimulates aldosteronogenesis in rat adrenal glomerulosa cells[206]. Plasma EKODE concentration correlates with aldosterone levels and BMI in humans[207]. In addition to oxidized fatty acid-induced aldosteronogenesis, it has been shown, that human adipocytes secrete potent aldosterone-releasing factors (ARFs) that induce aldosterone synthase expression and aldosterone production in human adrenal adrenocortical cells[208-209]. In addition to MR activation

by the increased aldosterone levels in obesity, the MR might be activated by increased ROS levels in obesity. Indeed, it has been demonstrated in a rat model of mild hypertension that ROS-activated MR enhances cardiac NADPH oxidase activity and dysfunction, [210].

Taken together, oxidized fatty acids as EKODE and so far unidentified ARFs, produced by the WAT induce adrenal aldosterone synthesis and in turn, increase aldosterone levels in obesity. Besides an aldosterone-mediated MR activation in obesity, the adipose tissue derived ROS in obesity might as well induce MR activation in aldosterone-sensitive tissues and contribute to the pathophysiological changes that are associated with obesity (Figure 14)[211].

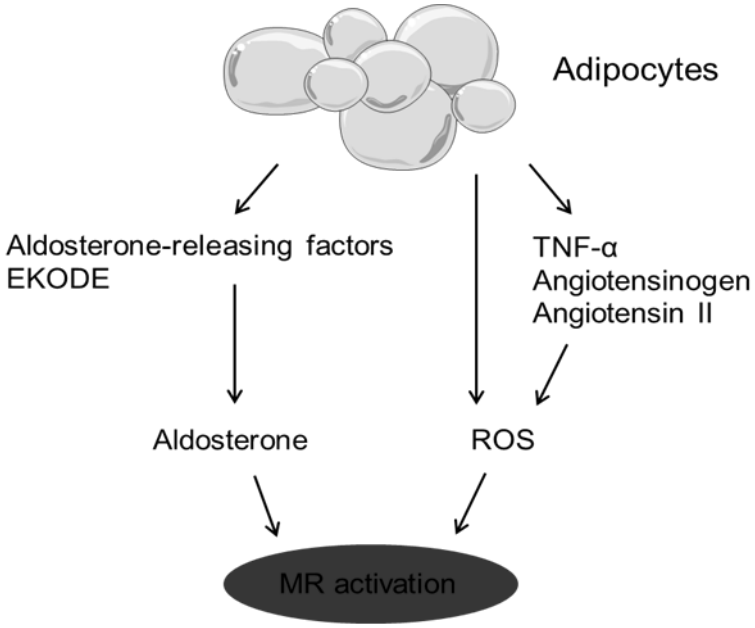


**Figure 14. The mechanism for MR-activation in obesity.** (Figure adapted from[211].)

The enzyme 11β-HSD1 plays a crucial role in determining the intracellular glucocorticoid levels[173]. Interestingly, activity and expression of 11β-HSD1 is enhanced in obese adipose tissue[212-213] and is associated with the development of obesity and metabolic syndrome[214-215]. Since adipocytes express a functional MR that can bind glucocorticoids with equal affinity as aldosterone, the increased glucocorticoid levels may also account for MR-mediated effects in adipocytes[216-218].

MR blockade in obesity decreases oxidative stress in WAT that might have positive effects on adipocytes dysfunction and, in turn insulin resistance[216]. Furthermore, MR antagonism attenuates obesity-induced expression of 11β-HSD1 as well as pro-inflammatory factors like TNF-α, MCP-1, PAI-1 and macrophage infiltration in the WAT[216,219]. In addition, MR blockade

reverses obesity-induced gene repression of adiponectin and PPAR-y (peroxisome proliferator-activated receptor-y) in adipose tissue[216,219]. Both adiponectin and PPAR-y are known to increase insulin sensitivity[220-221]. Thus, obesity-induced MR activation in the WAT enhances ROS generation and inflammation that might contribute to the development of obesity-related insulin resistance[217].

Indeed, various publications support an important role of aldosterone in the development on insulin resistance. A low insulin sensitivity in healthy subject can be correlated with increased aldosterone plasma levels[222]. Furthermore, hyperaldosteronism-induced insulin resistance can be successfully attenuated by MR antagonism[223]. Experiments in rats have shown that aldosterone administration diminishes insulin responsive glucose transporter (GLUT) 4 expression in skeletal muscle, as well as insulin-responsive GLUT 2 in the liver. These observations may account for skeletal muscle and hepatic insulin resistance in aldosterone-infused mice[224]. Studies in genetically obese mice revealed that MR antagonism improved glucose tolerance and decreased fasting levels of trigylcerides[216,219]. Furthermore, aldosterone degrades the insulin receptor substrate (IRS) 1 and IRS2 in cultured adipocytes. This degradation is induced by an glucocorticoid-receptor-mediated production of ROS and results in impaired insulin-induced glucose uptake[225].

Taken together, high aldosterone levels in obesity may not only contribute to hypertension, but also to insulin resistance and other components of the metabolic syndrome such as hyperlidipdemia, inflammation and oxidative stress which contribute to cardiovascular disease.

### 3.3.5 Role of aldosterone in cardiovascular disease

MR blockade by spironolactone or eplerenone are efficient treatments of hypertension[196,198] and confers cardiovascular protection independent of its beneficial effects on diuresis and blood pressure. MR antagonists have been reported to reduce morbidity and mortality in patients with congestive heart failure [226-227] and exert beneficial effects on patients with systolic heart failure and mild symptomes[228]. Since these effects are out of proportion given the modest diuretic effect of MR antagonists, MR action seems to have direct effects on cardiovascular tissue. This is supported by the observation that patients suffering from primary aldosteronism experience more cardiovascular events than patients suffering exclusively from essential hypertension[229].

Therefore, most harmful effects of aldosterone seem to be blood pressure-independent and are most likely carried out by direct action on the vasculature. Indeed, the MR is expressed in both aortic endothelial cells and in vascular smooth muscle cells and its expression is enhanced in certain pathological conditions as hypertension[191].

### 3.3.6 Role of aldosterone in endothelial activation and atherogenesis

Under normal conditions, endothelial cells resist prolonged contact with blood leukocytes. However, when activated by various stimuli such as oxidative stress or proinflammatory cytokines, endothelial cells express adhesion molecules, thus facilitating attachment and migration of leukocytes in the vessel wall and promoting an inflammatory response[230-231].

Indeed, various studies could show the contribution of aldosterone to proinflammatory cardiac and vascular responses. In the heart, administration of aldosterone is associated with increased expression of proinflammatory markers such as ICAM-1, MCP-1, TNF-α, osteopontin and COX-2. Moreover, aldosterone induces accumulation of macrophages and lymphocytes, as well as proliferation of fibroblasts in the coronary arteries of rats[232-233]. Further, MR antagonists diminish MMP-2 expression and oxidative stress in a mouse model of left ventricular hypertrophy[234].

In the vasculature, MR antagonism decreases atherosclerotic lesions in mice by diminishing aortic ROS production, and MCP-1 expression without influencing blood pressure[235]. Furthermore, chronic MR antagonism attenuates aortic COX-2, IL-1β, IL-6 and TNF-α expression in normo- and hypertensive rats[236]. In coronary artery smooth muscle cells, aldosterone enhances proinflammatory and profibrotic changes by inducing the expression of lymphocyte chemoattractant factor IL-16 and cytotoxic T-lymphocyte–associated protein 4[169]. In vascular endothelial cells the MR promotes ICAM-1 expression and leukocyte adhesion [170]. Furthermore, aldosterone increases osteopontin and ACE expression in aortic endothelial cells, which may be involved in the development of endothelial dysfunction and vascular injury[162]. Taken together, aldosterone induces cardiac and vascular inflammation via the vascular MR.

### 3.3.7 Role of aldosterone in endothelial dysfunction

Endothelial dysfunction is the initial step in the development of atherosclerosis and is characterized by a shift of the actions of the endothelium toward impaired vasodilation, a proinflammatory and prothrombotic state[237]. Several studies report that aldosterone contributes to endothelial dysfunction: Studies with young healthy subjects have shown that acute administration of aldosterone induces endothelial dysfunction, without affecting blood pressure[238]. A study with normotensive and hypertensive rats revealed that aldosterone administration induces endothelial dysfunction independent of blood pressure[239]. Furthermore, two different models of atherosclerosis in nonhuman primates and rabbits showed that blockade of the MR using Eplerenone ameliorates endothelial function without changing blood pressure or total cholesterol[240-241]. Endothelial cells respond to aldosterone with an increase in cell volume by intracellular accumulation of organic matter. This induces swelling of the

endothelial cells and stiffness of the vasculature[242-243]. These observations suggest that aldosterone influences endothelial function via non-hemodynamic mechanisms: One important pathway can be the decreased bioavailability of NO, leading to impaired endothelial-relaxation of the vessel. The decrease in NO can be attributed to decreased amount of NO production and increased NO inactivation by ROS[244].

Experiments in rats demonstrate that MR antagonism using eplerenone prevents a decrease in eNOS expression in hypertension[245] and heart failure[246]. Aldosterone diminishes eNOS through at least two different mechanisms. First, aldosterone reduces NO production by dephosphorylation of the N-terminal 1177 serine residue of eNOS, resulting in inactivation of the enzyme[247]. Second, aldosterone reduces expression of the eNOS cofactor BH4 leading to uncoupling of eNOS function[247]. This means that in absence of BH4, eNOS produces ROS instead of NO[248]. Moreover, aldosterone has been shown to inhibit iNOS in vascular smooth muscle cells via enhance IL-1β signalling[249].

On the other hand, aldosterone can increase oxidative stress by both increasing ROS production and reducing ROS scavenging capacity in the cells. It has been shown, that aldosterone impairs endothelial function by decreasing glucose-6-phosphate dehydrogenase (G6P-DH) expression via cyclic AMP-response element modulator (CREM) induction and subsequent cAMP inhibiton. Overall, the decreased G6P-DH expression and activity impairs its reducing properties concerning oxidation of $NADP^+$ to NADPH and results therefore in increased oxidative stress[171]. Moreover, in a rat model of heart failure, spironolactone added to an ACE inhibitor improves endothelium-dependent aortic relaxation, most likely by suppression of superoxide formation[250].

Aldosterone administration has been shown to increase superoxide production in cultured ventricular myocytes[251], rat VSMCs[252] and rat aortic endothelial cells[218,253]. Furthermore, aldosterone-induced ROS production has been observed in different pathological situations since aldosterone enhances and eplerenone attenuates expression of NADPH oxidase subunits and oxidative stress in hypertensive rats[254], rat hindlimb ischemia[255] , a mouse model of left ventricular hypertrophy [234] as well as in different animal models of atherosclerosis[234-235,240,256-257]. Along these lines, MR activation contributes to AngII-mediated activation of the NADH oxidase and superoxide production in the heart and the aorta[258-260]. Experiments in rats revealed, that exogenous aldosterone administration induces p22phox and gp91phox expression through an MR-dependent pathway whereas it induces p47phox expression through an MR and $AT_1$-receptor dependent pathway[254]. Apart from these aldosterone-induced changes in pro-oxidative systems, it has been shown that MR antagonism increases antioxidant capacity by enhancing hepatic antioxidant gluthatione in hypertensive rats[236].

Beyond its direct pro-oxidative effects on the vasculature, aldosterone enhances the pro-oxidative effects of AngII that activates the NADPH oxidase in VSMC, monocytes, macrophages and endothelial cells[67,261]. Antagonism of the MR ameliorates AngII-induced superoxide production in the heart and the aorta[258-260] and attenuates endothelial dysfunction in AngII infused rats, either[258]. Besides its action on AngII effects, aldosterone enhances ACE expression in rat aortic endothelial cells and cardiomyocytes[262-263] as well as $AT_1$ receptor expression in vascular smooth muscle cells[264]. This suggests a viscous cycle in which AngII, through the $AT_1$ receptor stimulates the production of aldosterone. This, in turn, leads to an increase of tissue ACE activity and an additional increase in AngII-enhanced oxidative stress[265].

An additional mechanism that contributes to aldosterone-induced endothelial dysfunction is the exaggerated production of vasoconstrictor factors. Indeed, aldosterone also stimulates endothelial dysfunction through activation of COX-2 in normotensive and hypertensive rats. Prostacyclin seems to be the main factor contributing to endothelial dysfunction in hypertensive rats, whereas other prostanoids such as prostaglandin $E_2$ and thromboxane appear to be involved in endothelial dysfunction under normotensive conditions[239,266].

Taken together, various studies demonstrated the involvement of aldosterone in the development of endothelial dysfunction, on the one hand by decreasing NO bioavailability and on the other hand, by enhancing oxidative stress, RAS system activity and prostaglandin production. However, the role of the increased aldosterone levels in obesity-induced endothelial dysfunction needs further investigations. Especially, the role of the endothelial MR, directly exposed to the increased aldosterone in the plasma in obesity, is unknown. Its activation might be of major importance since the endothelial MR is directly exposed to circulating aldosterone and has been shown to induce signaling pathways that contribute to endothelial dysfunction.

## 3.4 Aim of the study

Based on the published observations described above, we hypothesized that

- **Increased aldosterone in obesity induces endothelial dysfunction that is associated with an enhanced expression of proinflammatory and prooxidative factors in aortic endothelial cells**
- **The endothelial MR plays a critical role in mediating these effects to the arterial wall.**

Our overall strategy is to assess the role of aldosterone in the context of obesity and endothelial dysfunction. We will test above hypotheses by fulfilling the following specific aims:

**Aim 1 - Characterize effects of aldosterone/MR on obesity-induced endothelial dysfunction and aortic endothelial cell mRNA expression pattern.**

Test the role of aldosterone and MR in obesity-induced endothelial dysfunction and aortic endothelial cell mRNA expression using a pharmacological approach. Comparison of 20-weeks old *C57BL/6* mice kept on a normal-fat (ND), a high-fat diet (HFD) or a high-fat diet containing the mineralocorticoid antagonist Eplerenone (HFD EPL) for 14 weeks.

**Aim 2 - Test effects of endothelial MR ablation on obesity-induced endothelial dysfunction.**

Assess the role of the endothelial MR in obesity-induced (*endogenous* aldosterone-mediated) endothelial dysfunction by using conditional knockout mice (CreLoxP system). Comparison of 20-weeks old endothelial cell MR knockout out mice (*EC MR KO*) and their corresponding littermates (*MR WT*), kept on a ND or HFD for 14 weeks.

**Aim 3 - Determine effects of endothelial MR deletion on aldosterone-induced endothelial dysfunction and aortic endothelial cell mRNA expression pattern.**

Investigate the role of the endothelial MR in *exogenous* aldosterone-induced endothelial dysfunction and characterize aortic endothelial cell expression pattern. Comparison of 17-20-weeks old *EC MR KO* and *MR WT* mice, infused with aldosterone for 2 weeks

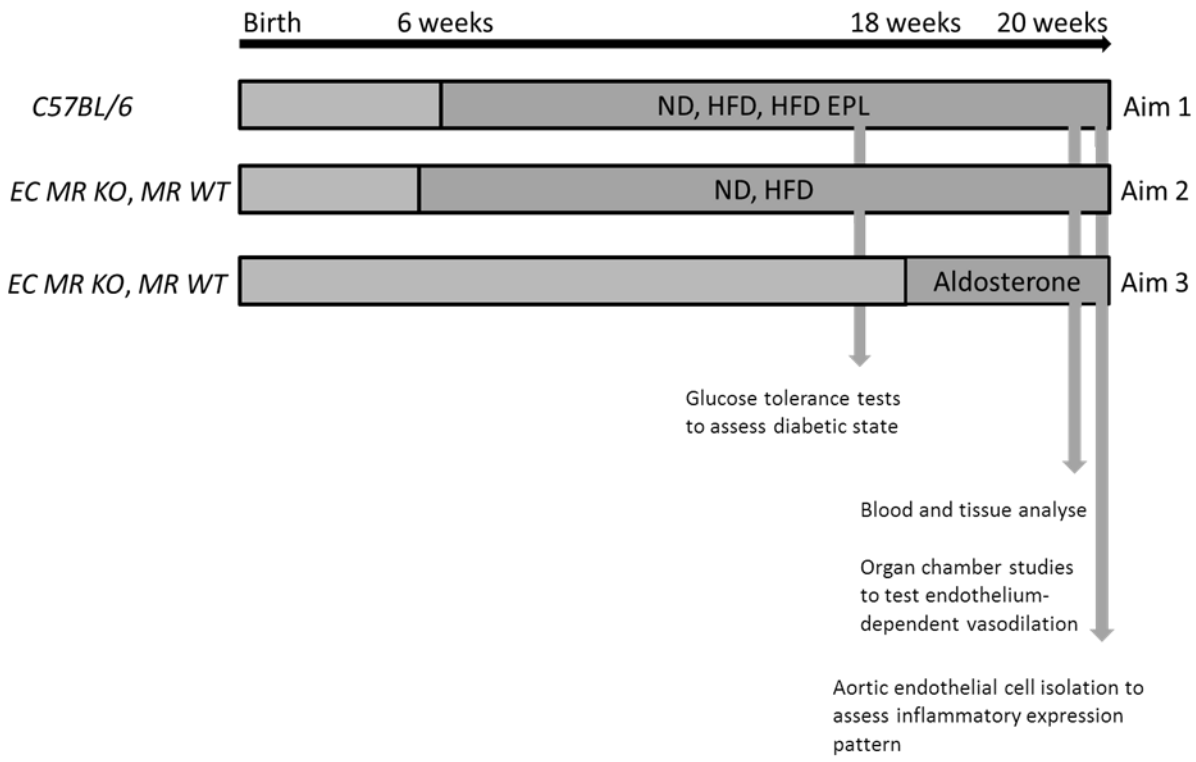


**Figure 14. Experimental protocols for *in vivo* and *ex vivo* experiments.** Concerning aim 1 and 2, in the 12th week of diet exposure, glucose tolerance tests were performed. After recovery phase of 2 weeks, mice were euthanized and blood plasma, cytokine expression in the WAT as well as endothelial function were analyzed. In addition, aortic endothelial cells were isolated in Aim I. Except glucose tolerance, all read-outs performed in Aim 1, were performed in Aim 3, as well.

# 4 Material and Methods

## 4.1 Mouse work

### 4.1.1 *C57BL/6* mice

*C57BL/6* mice were obtained from The Jackson Laboratory at the age of 6 weeks.

### 4.1.2 Conditional endothelial cell MR KO (*EC MR KO*) mice

The Cre-loxP system is used to generate conditional knockout animals. This mechanism is a special type of site-specific DNA recombination that leads to a tissue- or cell-specific inactivation of the gene of interest. The Cre-loxP system was developed in the 1980s[267-268] and is patented by the American chemical company DuPont.

It is a specific recombination system that is characterized by the expression of the P1 bacteriophage enzyme site-specific Cre recombinase (Cyclization Recombination) that recognizes and cuts 2 consensus 34 base pair DNA recognitions sites (*lox P* sites) that flank each side of a critical portion of the target gene that should be excised. Since the Cre recombinase and the *loxP* sites are not ubiquitously expressed in the mammalian genome, they must be introduced using transgenic mouse technologies.

To do so, two different transgenic mouse strains are generated. One mouse strain is supposed to express the gene of interest flanked with *lox P* sequences. Therefore, the transgenic sequence is constructed from the DNA coding sequence of the gene of interest, surrounded by ***loxP*** sites and introduced into embryonic stem cells that derived from the inner cell mass of a mouse blastocyst. Homologous recombination occurs in the embryonic stem cells leading to replacement of the target host gene with the modified gene copy.

Since the second strain of mice should express the Cre recombinase under the control of a cell-specific promoter, the coding sequence for Cre, controlled by the desired promoter, is introduced into specific cells in late stage embryos of a mouse line.

After generating the 2 transgenic mouse strains, they are crossbred to produce the Cre-loxP strain[269]. Homologous recombination between the *lox P* sites occurs in cells/tissue that express the Cre recombinase. This leads to a specific gene inactivation in a certain tissue. In cells not expressing the Cre recombinase, the promoter is inactive, the gene is preserved and can be expressed (Figure16).

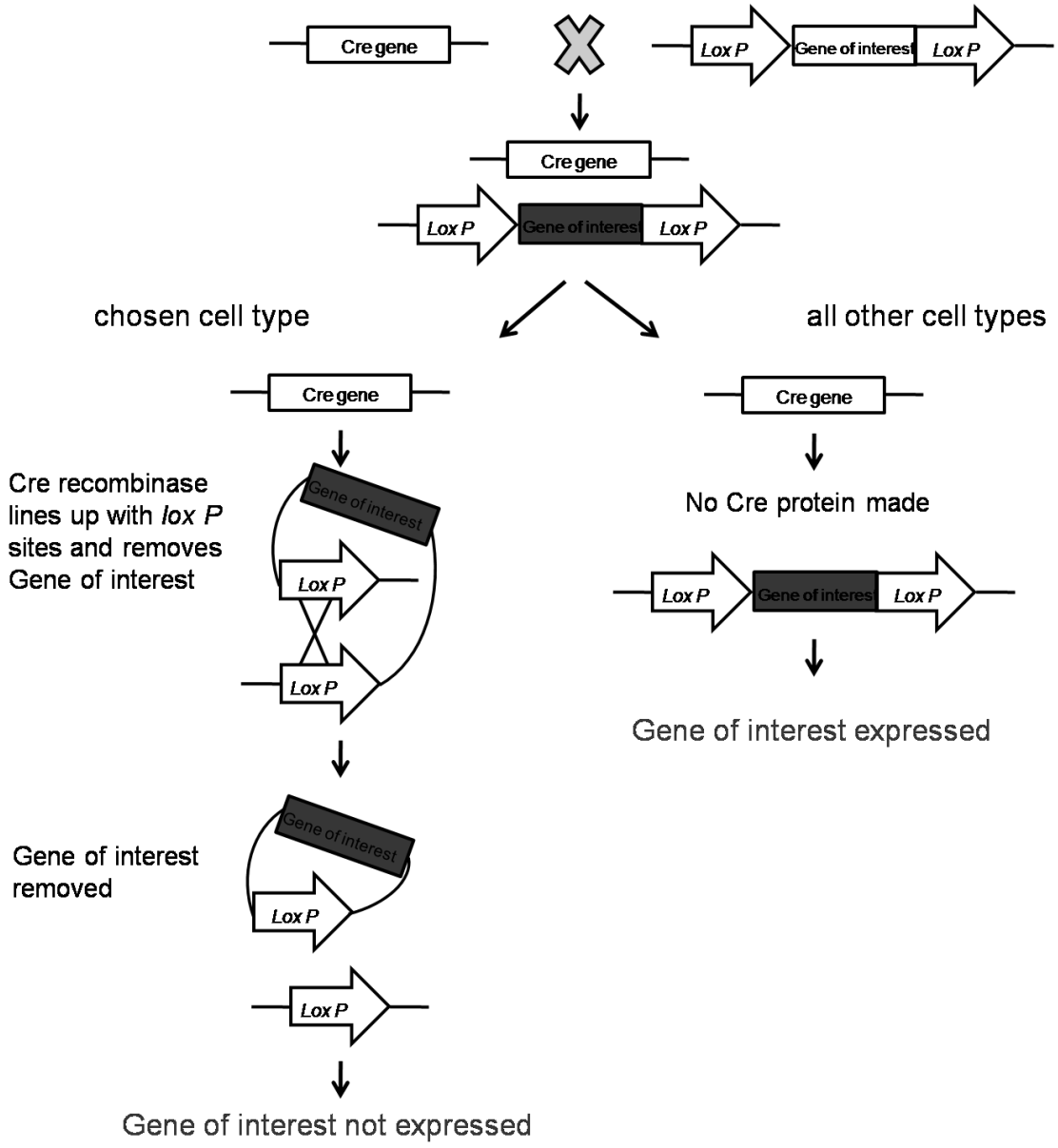


**Figure 16. Cre-loxP system.** Crossbreeding of the Cre recombinase transgenic mouse strains with a mouse strain, containing *lox P* sites that flank the gene of interest, results in the removal of the gene of interest in all cells expressing the Cre recombinase. All other cell types are unaltered. (Figure adapted from[270].)

To generate endothelial cell-specific MR KO mice we crossed *MR$^{flox}$* mice with *Tie2Cre* mice. The *MR$^{flox}$* mouse (B6.129P(FVB/N)-Nr3c2$^{tm\#Gsc}$) was kindly provided by the lab of Günther Schütz (German Cancer Center, Heidelberg). This mouse possessed a modified MR allele (MR$^{flox}$ allele), in which exon 3, encoding the first zinc finger of the MR DNA binding domain, was flanked by loxP sites[271]. Therefore, CreLox recombination should result in deletion of exon 3 and thereby prevent binding of MR to the DNA. In turn, MR-dependent transcription should be abolished.

Mice possessing the Tie2Cre transgene were obtained from the Jackson Laboratory (strain name: B6.Cg-Tg(Tek-cre)12Flv/J, stock number: 004128). The Cre recombinase expression in these mice was driven under the control of the endothelial-specific receptor tyrosine kinase promoter/enhancer (Tie2 or Tek). Tie2 is a receptor tyrosine kinase that binds angiopoietin-1 and angiopoietin-2 and is expressed in all endothelial cells. This allowed the expression of an active Cre recombinase in endothelial cells during embryogenesis and adulthood[272]. The *Tie2* promotor has also been detected in hematopoietic cells. This might lead to an undesirable Tie2-Cre recombination in the hematopoietic lineage[273] with MR deletion in the corresponding cells.

To generate endothelial cell-specific MR KO mice (*EC MR KO*) that were officially named B6.129P(FVB/N)-Nr3c2$^{tm4Gsc}$, *MR$^{flox}$* mice were crossbred with *Tie2Cre* mice.

In turn, cohorts of *MR WT* (*MR$^{flox}$/WT*) and *EC MR KO* (*MR$^{flox}$/Tie2Cre*) mice were bred by crossing *MR WT* and *EC MR KO* animals. Due to unspecific activation of Cre recombinase expression by the Tie2 promotor in the female germline[274], male mice were used to transmit the Tie2Cre transgene. This avoided undesirable Cre recombinase activity in cells except from endothelial cells and eventually hematopoietic cells. Genotyping was performed to detect the presence and of the floxed and deleted alleles[271] as well as the presence of the Tie2Cre transgene.

## 4.1.3 Genotyping

### 4.1.3.1 DNA preparation from the mouse tail

A 2 mm long piece of mouse tail was incubated in 200 µL NID (non ionic detergent) buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2 mM $MgCl_2$, 0.1mg/ml gelatine, 0.45% Tween20, 0.45% NP40) that contained 2 µL proteinase K (10 mg/mL). The mixture was incubated overnight at 56°C and afterwards inactivated at 96°C for 10 min.

### 4.1.3.2 *$MR^{flox}$* genotyping

The PCR reaction mix consisted of 1µl of each primer (10 µM), 0.4µl dNTPs (10 mM), 2µl reaction buffer (10x), 0.2 µl $Mg_2Cl$ (50 mM), 12.4 µl $ddH_2O$ and 0.2µl DNA polymerase (5 U/µl) (Eurobiotaq, Eurobion). 2 µl of DNA isolated from mouse tail was added to the PCR reaction mix.

| Primer (mouse) | 5` → 3` |
|---|---|
| MRflox fwd | CTG GAG ATC TGA ACT CCA GGC T |
| MRflox rev | TAG AAA CAC TTC GTA AAG TAG AGC T |

PCR program:

| Temperature | Time | Cycles |
|---|---|---|
| 96°C | 10 min | |
| 95°C<br>63°C<br>72°C | 30 sec<br>2 min<br>2 min | 35 |
| 72°C | 10 min | |
| 4°C | ∞ | |

The PCR product was mixed with 2 µl loading buffer (10x). 7.5µl of the mixture was loaded on a 2% agarose gel (100 V, 1 h).

Size of MR wild-type allele: 285 bp; size of $MR^{flox}$ allele: 335 bp.

#### 4.1.3.3 *Tie2Cre* genotyping

The PCR reaction mix consisted of 1 µl of each primer (10 µM), 2 µl dNTPs (2.5 mM), 2.5 µl reaction buffer (10x), 2.5 µl DMSO, 11.5 µl $ddH_2O$ and 0.5 µl DNA polymerase (5 U/µl) (TaKaRa). 2 µl of DNA isolated from mouse tail was added to the PCR reaction mix.

| Primer (mouse) | 5` → 3` |
|---|---|
| Tie2Cre fwd | GCG GTC TGG CAG TAA AAA CTA |
| TieCre rev | GTG CAG CAT TGC TGT CAC TT |
| IL fwd | CTA GGC CAC AGA ATT GAA AGA |
| IL rev | GTA GGT GGA AAT TCT AGC ATC ATC |

PCR program:

| Temperature | Time | Cycles |
|---|---|---|
| 94°C | 3 min | |
| 94°C<br>60°C<br>65°C | 1 min<br>30 sec<br>30 sec | 40 |
| 65°C | 5 min | |
| 4°C | ∞ | |

The PCR product was mixed with 2 µl loading buffer (10x). 7.5 µl of the mixture was loaded on a 2% agarose gel (100 V, 1 h).

Size of Tie2Cre transgene: 100 bp; IL (positive control): 324 bp

### 4.1.4. Feeding experiments

Male animals at the age of 6 weeks were exposed to a normal-fat diet (ND; 10kcal% lard, 1.25% total cholesterol, D07121304, Research Diets, New Brunswick, NJ, USA), a high-fat diet (HFD; 60kcal% lard, 1.25% total cholesterol, D0712305, Research Diets, New Brunswick, NJ, USA) or a high-fat diet containing eplerenone (HFD EPL; 60kcal% lard, 1.25% total cholesterol, 200mg/kg/day Eplerenone, D09012902, Research Diets, New Brunswick, NJ, USA) for 14 weeks. The animals were housed in the animal facility of the University of Zürich, Irchel Campus. They were exposed to a 12hours day-night-cycle and given free access to water. Weight gain was monitored on weekly bases. All animal experiments were approved by the local animal committee (animal protocol 2007/175) and performed in accordance with our institutional

guidelines. Animals were euthanized with isoflurane (Abbott; Baar, Switzerland) inhalation for tissue harvesting.

### 4.1.5 In vivo aldosterone infusion

Alzet osmotic minipumps (Alza, Model 1002) were preloaded with 50 µg/kg/d aldosterone[171,275] or the corresponding vehicle polyethylenglycol 400 (Sigma-Aldrich) and placed in 0.9% saline over night before implantation.

17-20 weeks old male mice were anesthetized using isoflurane inhalation. For the subcutaneous implantation of the pump, an incision was made in the skin between the scapulae. Using a hemostat, a small pocket was formed by spreading the subcutaneous connective tissue. The osmotic pump was inserted into the pocket with the flow moderator pointing away from the incision and the skin incision closed by suture. Weight and general health signs were checked every day post-operative. After 14 days, mice were euthanized using isoflurane and experiments were performed.

### 4.1.6 Metabolic cages

Animals were adapted to metabolic cages (Tecniplast, Buguggiate, Italy) for two times two days with breaks in normal cages in between. Animals had free access to ND, HFD and HFD EPL and drinking water. Daily, urine output and body weights were measured. Urinary plasma aldosterone levels were measured by radioimmunoassay (DRG instruments).

## 4.2 Measurement of plasma parameters

### 4.2.1 Glucose tolerance tests

For glucose tolerance tests, animals were fasted for 14 hours over night. The tip of the tail was cut about 0.3-0.5 cm from the end, and then basal fasted glucose was measured from a drop of blood emanating from the cut using a standard glucometer (Accu-Check Aviva, Roche). 2 g/kg glucose was injected intraperitoneally and blood was drawn after 15, 30 45, 60, 90, 120 and 180 min from the tail vein to determine blood glucose levels. As a reference value, plasma glucose was measured in unfasted state, either.

### 4.2.2 Plasma aldosterone and renin measurements

For determination of plasma aldosterone concentration as well as and renin concentration and activity, blood was drawn from the right ventricle after euthanizing the mouse. Plasma was transferred into an $K_2$EDTA-coated vial (BD Microtainer #365975), centrifuged (14`000 rpm) at 4°C for 10minutes, and stored at -80°C. Plasma aldosterone was measured by radioimmunoassay (DRG instruments), renin activity and concentration as previously decribed[276] by our collaborator Jürg Nussberger (University of Lausanne).

## 4.3 Analysis of vascular function

Following euthanasia, abdomen and thorax of the mouse was opened, organs carfully removed and aorta was cut proximal to the aortic arch and the diaphragma. Paying attention not to harm or overstretch the aorta during the dissection procedure, connective tissue and periadventitial fat were carefully removed.

The vessel was cut into 8 aortic rings (2-3 mm long), of which each was immersed in a 95% $O_2$/5% $CO_2$ aerated organ chamber filled with modified Krebs-Ringer bicarbonate solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $NaH_2PO_4$, 1.2 mM $Na_2SO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM glucose, pH to 7.4) and hooked to an isometric force transducer (MultiMyograph).

After equilibration for 60 min, the rings were progressively stretched to 1 g (5 mN). Contraction of the rings was assessed by incubation with 80 mM KCl. After 3 min of incubation, KCl was flushed off and rings were equilibrated in Krebs-Ringer bicarbonate solution for another 30 min. In turn, rings were again stepwise stretched to their optimal passive resting tension (2 g = 10 mN). Maximal contraction of the rings was assessed by repeated incubation with 80mM KCl for 3 min. Again, KCl was flushed off and rings were equilibrated in Krebs-Ringer bicarbonate solution for 30 min.

To assess endothelial function, aortic rings were pre-constricted with norepinephrine (NE), whereas the NE-induced contraction was supposed to be 50-70% of the maximal contraction induced by KCl. Following the contraction, endothelium-dependent relaxation was induced by acetylcholine in a cumulative fashion (Ach, $10^{-9}$ to $10^{-4}$ M; Sigma-Aldrich) (Figure 17). Afterwards, the solution was flushed off and rings were equilibrated for 30 min.

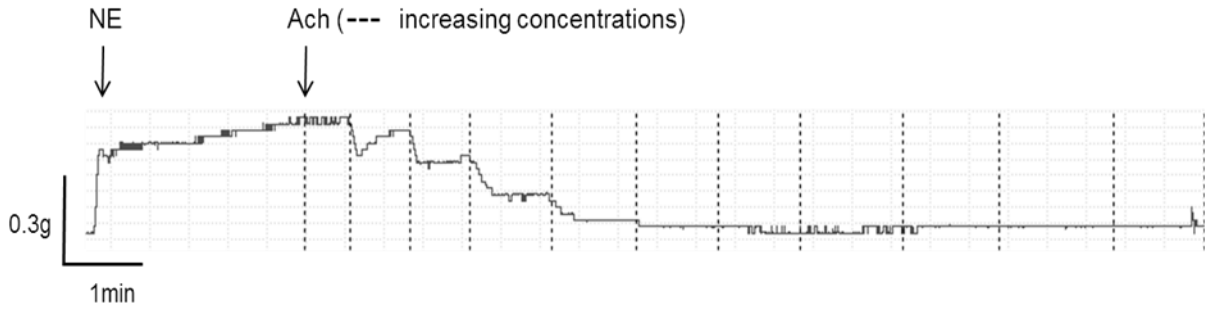


**Figure 17. Original vascular reactivity trace using an organ chamber.** Aortic rings were clamped into the organbath, pre-constricted with norepenephrine and afterwards relaxed with acetylcholine (Ach, $10^{-9}$ to $10^{-4}$ M) in a cumulative fashion to assess the endothelium-dependent relaxation of the aorta.

To measure endothelium-independent relaxation aortic rings were again pre-constricted with NE (30%-70% of KCl-induced contraction) and relaxed by using the external NO-donor sodium nitroprussid (SNP, $10^{-9}$ to $10^{-4}$ M; Sigma-Aldrich) in a cumulative fashion. Relaxations to the agonist in aortic rings were given as percent pre-contraction induced by NE. As a control, concentration-dependent contractions were induced by using NE ($10^{-9}$ to $10^{-4}$ M; Sigma-Aldrich) in a cumulative fashion. Contractions to NE were expressed as percentage of 80 mM KCl-induced contractions.

Whereas 7 aortic rings were treated in parallel as described above, one aortic ring was pre-incubated with indometacine ($5x10^{-6}$ M) 30 min before performing each dose-response curve.

## 4.4 Isolation of fresh aortic endothelial cell mRNA

To isolate fresh mouse aortic endothelial cells, I have established a protocol that allowed me to isolate the cells within 70 min after the death of the mouse (Figure 18).

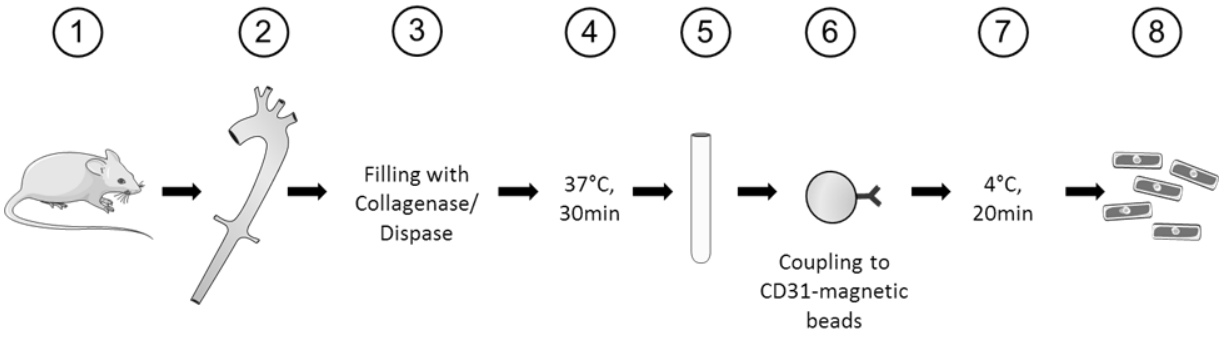


**Figure 18. Mouse aortic endothelial cell isolation.**

Immediately after euthanizing the mouse (Figure 18.1), the midline of the abdomen was incised and the thorax was open to expose heart and lung. The right atrium was cut and a whole body-perfusion from the left ventricle (100 mmHg) was carried using 10 ml ice-cold HBSS$^{Ca/Mg\ free}$ (Gibco). After dissection of the aorta from the aortic arch to the abdominal aorta, removal of connective tissue and periadventitial fat, the aortic branches and the aorta distal to the thoracic diaphragm were ligated with fiber (Figure 18.2). The aorta was then filled with a collagenase/dispase solution (4 mg/ml collagenase/dispase (Roche, #10269638001, lot#11730030), 147 pg/ml TLCK (#A17990100 BioChemica), 10 µg/ml DNAseI (Roche, #11284932001), 600 ng/ml bovine albumin (Sigma, #A8412), 6 mM HEPES (Gibco, #15630) diluted in 1x HBSS$^{Ca2+\ Mg2+\ free\ (w\ phenol\ red)}$ (Gibco) (Figure 18.3). After filling, the aorta was ligated proximal to the heart, excised and incubated for 30 min at 37°C to detach endothelial cells from the aortic wall (Figure 18.4). After the incubation time, cells were flushed out using 10 ml HBSS$^{Ca/Mg\ free}$, centrifuged for 5 min at 1`200 rpm and resuspended in 400 µl of 1 mg/ml bovine albumin (Sigma, #A8412), 10 mM HEPES (Gibco, #15630) diluted in HBSS$^{Ca2+\ Mg2+\ free\ (w\ phenol\ red)}$ (Gibco) (Figure 18.5).

During this procedure, an endothelial cell-specific antibody (purified rat anti mouse CD31, BD Pharmingen #557355) was precoupled to Dynabeads (sheep anti-Rat IgG, Dynal, Invitrogen #110.35) in a solution consisting of 1 mg/ml bovine albumin (Sigma, #A8412) and 2 mM EDTA in PBS $^{Ca2+\ and\ Mg2+\ free}$ (Gibco), pH 7.4 at 4°C for 2.5 h (Figure 18.6).

The detached aortic cells were purified by incubation of the cell fraction with dynabeads, pre-coupled to the CD31 antibody, for 20 min at 4°C (Figure 18.7). The purified aortic endothelial cells (Figure 18.8) were either stored at -80°C or directly used for RNA isolation using the Micro RNeasy Kit (Qiagen #74004). Since a small amount of cells was processed, 20 ng carrier RNA (16S and 23S-ribosomal, Roche #206938) was added to the cell lysate before homogenisation.

A MicroRNA Chip was performed to check concentration and integrity of the RNA. Good RNA quality (RNA integrity number above 6) allowed the amplification of the RNA by using the TransPlex Whole Transcriptome Amplification Kit (Sigma-Aldrich, #WTA1) and purification of the amplified RNA by using the PCR Purification Kit (Qiagen # 28104).

## 4.5 Isolation of tissue RNA

Harvested tissue was stored for 12 h at 4°C in RNAlater solution (Ambion, #AM7021),.The solution was then removed, samples were snap frozen in liquid nitrogen and stored at -80°C until further processing. Total RNA isolated from proximal aortae, epididymal, mesenteric and periadventitial white adipose tissue was extracted with TRIZOL (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, #27-9264-01).

## 4.6 Real-Time RT-PCR

Real-Time RT-PCR was performed using the SYBR®Green JumpStart™ Taq ReadyMix™ (SIGMA #S4438). The reaction mix consisted of 1 µl of each primer (10 µM), 12.5 µl PCR MIX (ready-to-use), 0.25 µl dye (ready-to-use), 8.25 µl $H_2O$ and 2 µl cDNA. For whole organ expression studies, 10 ng of cDNA and for aortic endothelial cell expression studies, 7.5 ng of cDNA per reaction were used.

| Primer (mouse) | 5` → 3` |
|---|---|
| S12 fwd | GAAGCTGCCAAAGCCTTAGA |
| S12 rev | AACTGCAACCAACCACCTTC |
| vWF fwd | CAGCATCTCTGTGGTCCTGA |
| vWF rev | GATGTTGTTGTGGCAAGTGG |
| S100A4 fwd | GGCCCTGGATGTAATTGTGTC |
| S100A4 rev | CACCTTCTGGAATGCAGCTTC |
| aSMA fwd | CTGACAGAGGCACCACTGAA |
| aSMA rev | CATCTCCAGAGTCCAGCACA |
| Hbb-b1 fwd | ATGGCCTGAATCACTTGGAC |
| Hbb-b1 rev | ACGATCATATTGCCCAGGAG |

| Primer (mouse) | 5` → 3` |
|---|---|
| MR fwd | AAGATCTGCTTGGTGTGTGGAG |
| MR rev | GCAGGACAGTTCTTTCTCCGAA |
| TNFα fwd | CCCTCACACTCAGATCATCTTCT |
| TNFα rev | GCTACGACGTGGGCTACA |
| MCP-1 | CCCAATGAGTAGGCTGGAGA |
| MCP-1 | TCTGGACCCATTCCTTCTTG |
| CD68 fwd | ACATTGTATTCCACCGCCAT |
| CD68 rev | GAGGTTGTGACGGTACCCAT |
| C1-inhibitor fwd | TGGACCAGGACGCAGCTGACA |
| C1-inhibitor 1 rev | TGGGTGCTGTGGCTGGTAGC |
| ICAM-1 fwd | GTCCGCTGTGCTTTGAGAAC |
| ICAM-1 rev | GCACCGTGAATGTGATCTCC |
| VCAM-1 fwd | ATATGACATGCTTGAGCCAGGGC |
| VCAM-1 rev | GTGTCTCCTTCTTTGACACTCTC |
| eNOS fwd | CTGGGTTTAGGGCTGTGCGGC |
| eNOS rev | CCAGGGATCCCAAGCAGCGTC |
| Prostacyclin Synthase fwd | GGCTCCTTGTCAGCGGGGGATA |
| Prostacyclin Synthase rev | CCGCGAGGGCTTCCGGATTC |
| COX-1 fwd | CTTCTCCACGATCTGGCTTC |
| COX-1 rev | GCTCGCAGGAAATAGCCACTC |
| COX-2 fwd | GCTGTACAAGCAGTGGCAAA |
| COX-2 rev | CCCCAAAGATAGCATCTGGA |
| SOD-1 fwd | TACTGATGGACGTGGAACCC |
| SOD-1 rev | GAACCATCCACTTCGAGCA |
| SOD-3 fwd | ATGGCTGAGGTTCTCTGCAC |
| SOD-3 rev | ACTCAGAGGCTCTTCCTCCG |
| Catalase fwd | ATGACAACCAGGGTGGTGCCCC |
| Catalase rev | CCTGGATGCGGGCCCATAGT |
| p22phox fwd | GTGTGAAACGTCCAGCAGT |
| p22phox rev | GTCATGGGGCAGATCGAGT |
| p47phox fwd | TCCTCTTCAACAGCAGCGTA |
| p47phox rev | CTATCTGGAGCCCCTTGACA |
| p40phox fwd | CCTGCCCACATTGCCAGCCA |
| p40phox rev | AGACCGGCAGGCTCAGGAGG |
| Rac-1 fwd | TTTTCCCCAGCTTTGGGTGGTGG |
| Rac-1 rev | TGGTCGTGTAACTGATGAGCAGGC |

qPCR program:

| Temperature | Time | Cycles |
|---|---|---|
| 95°C | 10min | |
| 95°C<br>55°C<br>72°C | 30 sec<br>1 min<br>30 sec | 40 |
| 95°C | 1 min | |
| 55°C | 30 sec | |
| 95°C | 30sec | |
| 4°C | ∞ | |

The expression of mRNA of genes of interest was calculated relative to the ribosomal S12 as reference housekeeping gene. Relative expression ratios were estimated as R = (2(Ct(reference) – Ct(test)). Ct: cycle number at the threshold, test: tested mRNAs (e.g. MR).

## 4.7 Immunofluorescence staining of von Willebrand factor (vWF)

Aortae were embedded in OCT and stored at -80°C. 0.4 µm thick slices were cut using a Cryostat and mounted on a microscope slide. After air drying for 15 min, slices were washed with PBS and fixed with 4% paraformaldehyde (PFA). Cryosections were blocked with 3% BSA / 0.1% Triton-X100 for 30 min and incubated at room temperature for 90 min with the primary antibody against vWF (rabbit anti-human) diluted 1:400 in 3% BSA / 0.1% Triton-X100. After washing, slices were incubated for 30 min at room temperature with conjugated secondary antibody (Texas Red, 594 goat-anti-rabbit IgG, Invitrogen, USA) diluted 1:500 in 3% BSA/0.1% Triton-X100. Slices were mounted with DAPI-fluorescence mounting media (Vectashield, Vector Laboratories, Inc., Burlingame, CA 94010) and images acquired using the Nikon Eclipse TE3000 inverted fluorescence microscope (Nikon AG Küsnacht, Switzerland) equipped with a DS-5M Standard CCD camera (Nikon) and the NIS-Elements software (Nikon). Images were further processed using Photoshop 7 (Adobe, San Jose, USA).

## 4.8 FACS analyses

Isolated aortic endothelial cells were incubated with a 0.5% BSA-PBS for blocking. After that, cells were incubated with a biotinylated primary antibody that recognizes Isolectin B4 (Vectorlab #B-1205) diluted 1:50 in 0.5% BSA-PBS for 30 min. After washing with PBS, cells were incubated with a secondary antibody, namely FITC-conjugated Streptavidine (eBiosience #11-4317-87) diluted 1:50 in 0.5% BSA-PBS for 30 min. Flow cytometry analyses were performed using the FACSCanto. Analyses were performed using the FlowJo software.

## 4.9 Statistics

Data were expressed as means ± standard error of the mean (SEM). Calculations were carried out using Microsoft Excel, while the graphs were created using GraphPad Prism™ Version 5.0 (GraphPad Inc.). Significance was tested with GraphPad Prism™ using students T-Test unpaired or one-way ANOVA. Statistical significance was accepted at $P < 0.05$.

# 5 Results

## 5.1 MR antagonism attenuates obesity-induced endothelial dysfunction by modulating inflammation and enzymes, associated with prostanoid and ROS production

### 5.1.1 MR antagonism attenuates obesity-associated glucose intolerance

To address the contribution of endogenous aldosterone in the development of endothelial function, we induced obesity by exposing 6-week-old male *C57BL/6* mice to a high-fat diet in the absence (HFD) or presence of eplerenone (HFD EPL) for 14 weeks. Control mice were fed a normal diet (ND). Compared to lean mice, HFD feeding resulted in a significant increase in relative body weight and total epididymal WAT, an established marker for obesity in male mice (Figure 19 A, B). Feeding HFD EPL led to the same weight gain in mice as feeding with HFD in absence of EPL (Figure 19A). Therefore, eplerenone has no effect on the degree of diet-induced obesity in mice.

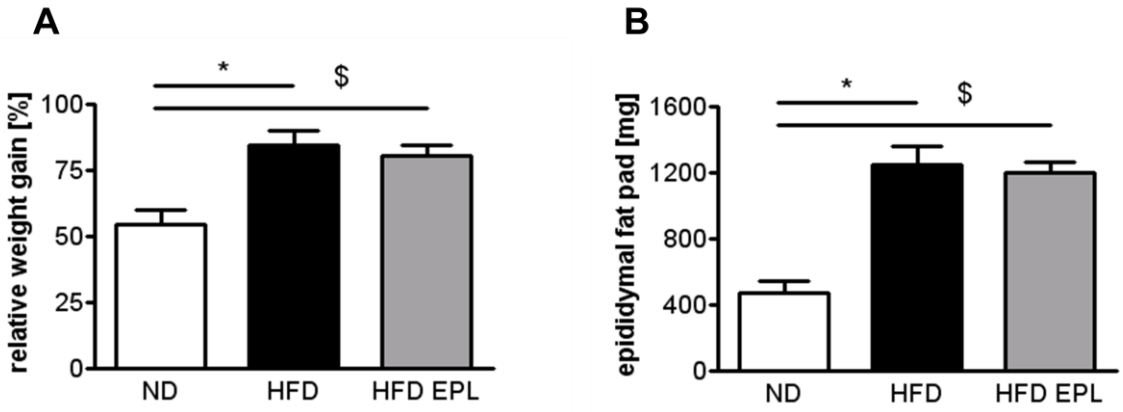


**Figure 19. Increased weight gain in lean and diet-induced obese *C57BL/6* mice with or without pharmacological MR antagonism.** C57BL/6 mice were treated with ND, HFD or HFD EPL. After 14 weeks (**A**) relative weight gain and (**B**) epididymal fat pad mass were measured. Data are mean±SEM; n=6–10. **P*<0.05 for ND vs HFD; \$*P*<0.05 for ND vs HFD EPL.

Since eplerenone can exert beneficial effects on insulin resistance[216] we assessed the metabolic state of the ND, HFD and HFD EPL fed mice, 12 weeks after beginning of diet exposure. This allowed the mice to recover for 2 week after the glucose tolerance tests, before using them for the actual primary end-point experiments. Blood glucose was similar in all 3 groups of mice in the non-fasted state (Figure 20A), whereas glucose levels were significantly increased in obese fasted mice (Figure 20B). EPL treatment of obese mice reduced this hyperglycemic state, non-significantly (Figure 20B). To characterize the glucose tolerance more in detail, we performed blood glucose tolerance tests (Figure 20C). Indeed, we observed an impaired glucose tolerance in obese mice. Eplerenone treatment in obesity restored the impaired glucose tolerance 180 min after the glucose load. We could conclude that EPL attenuates hyperglycemia as well as blood glucose tolerance in obesity.

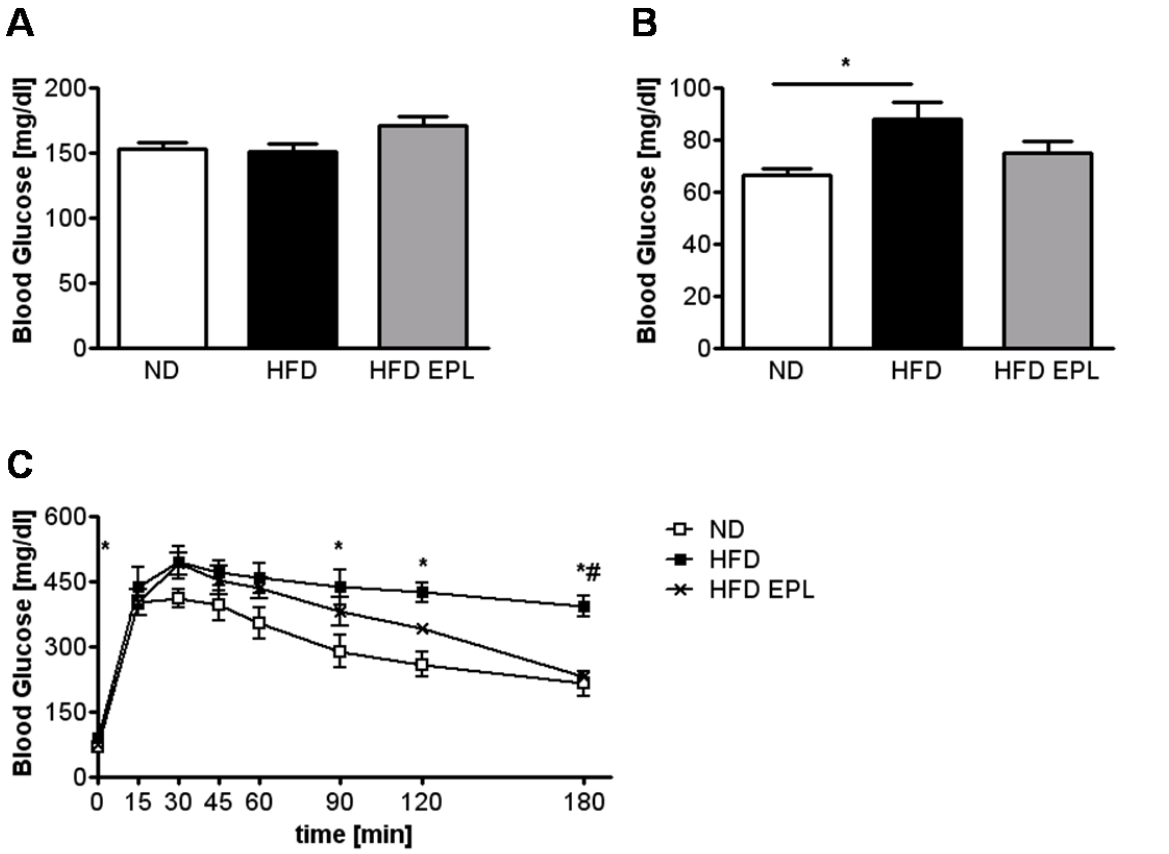


**Figure 20. Impaired glucose tolerance in diet-induced obese *C57BL/6* mice is attenuated by MR antagonism.** *C57BL/6* mice were treated with ND, HFD or HFD EPL for 14 weeks. After 12 weeks of diet blood glucose levels were determined (**A**) under fed state (**B**) after fasting for 16h over night and (**C**) in a time course after injection of 2 g glucose/kg bodyweight. Data are mean±SEM; n=6–10. **P*<0.05 for ND vs HFD; #*P*<0.05 for HFD vs HFD EPL.

### 5.1.2 Increased plasma aldosterone concentration in obesity

To characterize the putative activation of components of the RAAS in our mouse model of obesity, we assessed renin concentration and activity as well as plasma and urine aldosterone levels. Considering the circadian alterations of the RAAS[277-278], we decided to take the mouse plasma always at the same time of the day, at 9 a.m. Renin concentrations and activity remained unaltered in mice fed a HFD in absence or presence of EPL compared to lean mice (Figure 21A, B), whereas aldosterone concentrations were significantly higher in obesity (Figure 21C). These observations indicate that obesity increases plasma aldosterone in a renin-independent way. Additionally, we observed an even further increase in plasma aldosterone when EPL was administered. Since an unspecific cross-reaction of the Aldosterone-RIA with the EPL in the mouse plasma could not be excluded, we performed an additional experiment to test if the increased aldosterone concentration in the HFD EPL group is a false positive result. It has been shown in humans that an oral single dose of 100 mg EPL results in maximal 1.72 µg/ml EPL in the plasma[279-280]. Since we administered 8 mg EPL/day via diet to our mice, we expected EPL in the concentration in the plasma approximately 96`000 ng/l. Therefore, we administered either $10^5$ ng/l or $10^7$ ng/l EPL to mouse plasma and measured aldosterone concentration. In fact, we could show that aldosterone levels are not falsely increased in mouse plasma, containing EPL (Figure 21D). We conclude that the Aldosterone-RIA recognizes aldosterone specifically and that EPL treatment increases aldosterone release in obesity, most likely due to a blocked MR-dependent regulatory feedback loop.

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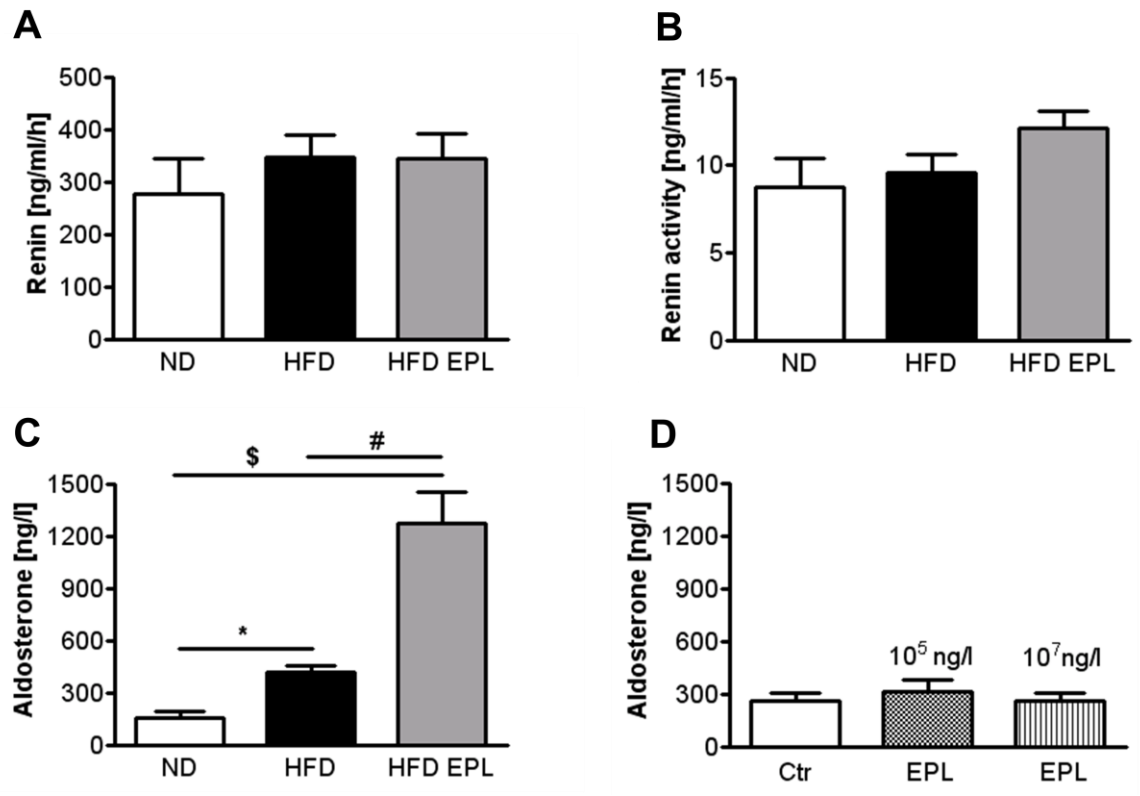


**Figure 21. Unaltered renin levels and increased plasma aldosterone levels in diet-induced obese *C57BL/6* mice.** *C57BL/6* mice on ND, HFD and HFD EPL (n=8-10) were analyzed concerning **(A)** plasma renin concentration, **(B)** plasma renin activity and **(C)** plasma aldosterone concentration. **(D)** Aldosterone concentrations were measured in plasma of *C57BL/6* containing 100`000 ng/l or 10`000`000 ng/l EPL. Data are mean±SEM; n=6–10. **P*<0.05 for ND vs HFD; #*P*<0.05 for HFD vs HFD EPL;$*P*<0.05 for ND vs HFD EPL.

To characterize the release of aldosterone in obesity in more detail, we performed experiments with metabolic cages and sampled the urine of ND and HFD fed mice every 24 hours. Surprisingly, we observed decrease aldosterone levels in the urine of obese mice (Figure 22A). In order to exclude, that the decreased aldosterone levels in obesity arise a faster degradation of aldosterone in its tetra-hydro-from, which could not be detected by the Aldosterone-RIA, we tried to assess the activity of the aldosterone-degrading reductase in an indirect manner. Aldosterone is degraded to its dehydro- as well as tetrahydro-metabolites by the same 5β-reductase as corticosterone. Since our collaborator Brigitte Frey (Inselspital, Bern) was able to assess the concentration of the corticosterone degradation products tetrahydro-corticosterone (TBH), 5α-tetrahydro-corticosterone (5$^{\alpha}$-THB) and 11-dehydro-corticosterone (THA) in the urine of the mice (Figure 22B), we hoped to receive information about the activity of the 5β-reductase. Unluckily, the amount of urine we had available was too small to measure the degradation products in each sample. Therefore, we are not able to explain the reason for the decreased urinary aldosterone levels in obesity.

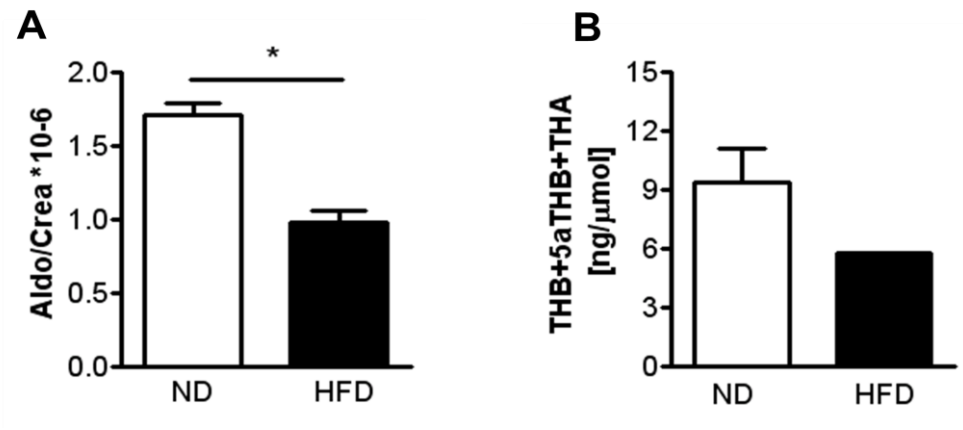


**Figure 22. Decreased urinary aldosterone and corticosterone metabolites in diet-induced obese *C57BL/6* mice.** *C57BL/6* mice on ND, HFD were analyzed concerning **(A)** urinary alosteron/creatinine and **(B)** corticosterone degradation products tetrahydro-corticosterone (TBH), 5α-tetrahydro-corticosterone (5$^{\alpha}$-THB) and 11-dehydro-corticosterone (THA). Data are mean±SEM; n=1–5. **P*<0.05 for ND vs HFD.

In a next step, we decided to investigate whether MR mRNA expression levels were changed in aortic or in epididymal WAT upon of ND, HFD and HFD EPL feeding, respectively. Compared to lean mice, MR mRNA expression tended to be increased in obese mice in the aortae as well as in the epididymal and mesenteric WAT. EPL HFD treatment further increased MR mRNA expression in these tissues (Figure 23A, B). The observations concerning increased plasma aldosterone as well as tissue MR mRNA expression suggest an important role of aldosterone-induced MR signaling in obesity.

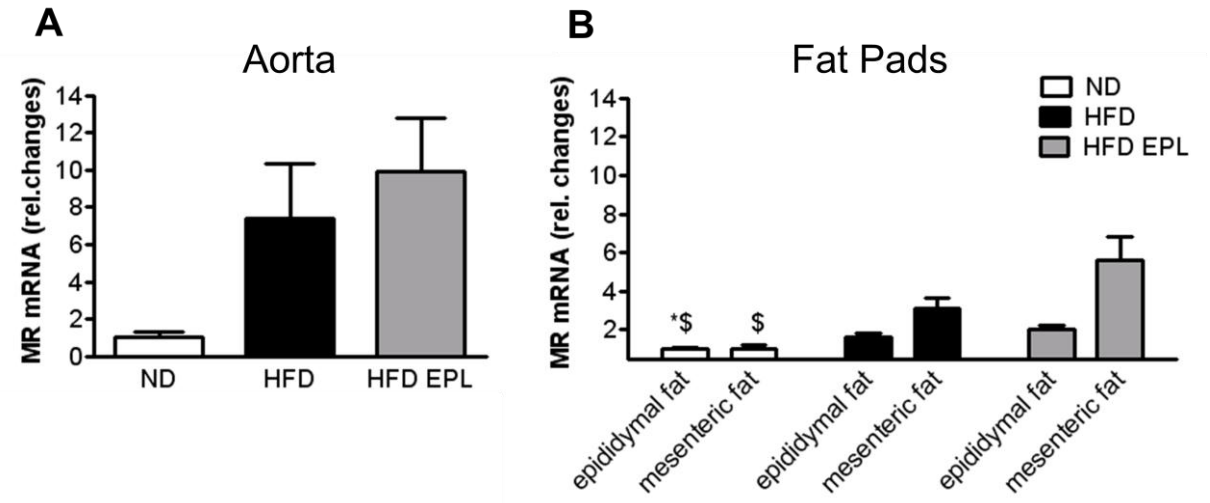


**Figure 23. Tissue MR expression is increased in obesity and further elevated by MR antagonism.** *C57BL/6* mice on ND, HFD and HFD EPL (n=8-10) were analyzed concerning **(A)** MR mRNA in aortic lysates and **(B)** MR mRNA in epididymal and mesenteric fat pads, standardized to S12 and normalized to ND. Data are mean±SEM; n=6–10. **P*<0.05 for ND vs HFD; $*P*<0.05 for ND vs HFD EPL.

### 5.1.3 MR antagonism attenuates obesity-induced proinflammatory changes in WAT

It has been demonstrated, that MR antagonism can reverse proinflammatory cytokine expression in the adipose tissue of genetically obese mice[219]. Along that line, we investigated whether the inflammatory state in diet-induced obese mice was altered by MR antagonism, either. To do so, we isolated the epididymal, mesenteric and periadventitial fat pads of *C57BL/6* mice on ND, HFD or HFD EPL and extracted mRNA for qPCR analyses.

The epididymal WAT of obese animals showed elevated mRNA levels of macrophage-specific glycoprotein CD68, suggesting an increased macrophage infiltration in this tissue (Figure 24A). Moreover, mRNA levels of proinflammatory cytokines such as TNF-α and MCP-1 were increased in the epididymal and mesenteric WAT of obese mice compared with lean control mice (Figure 24A, B). Chronic treatment with EPL markedly reduced mRNA levels of TNF-α and MCP-1 in the epididymal and mesenteric, as well as CD68 mRNA expression in the epididymal WAT (Figure 24A, B). In addition, we observed an increased expression of CD68 in the periadventitial fat of obese mice, but no change upon EPL administration (Figure 24C). Due to a marked variation of results, a regulation of MCP-1 under HFD conditions in the periadventitial white adipose tissue is doubtful. Furthermore, TNF-α mRNA expression remained unaltered in this fat pad.

These data suggest that obesity-induced proinflammatory changes are most pronounced in the epididymal WAT. These inflammatory changes are diminished by MR antagonism in the epididymal, mesenteric and periadventitial WAT.

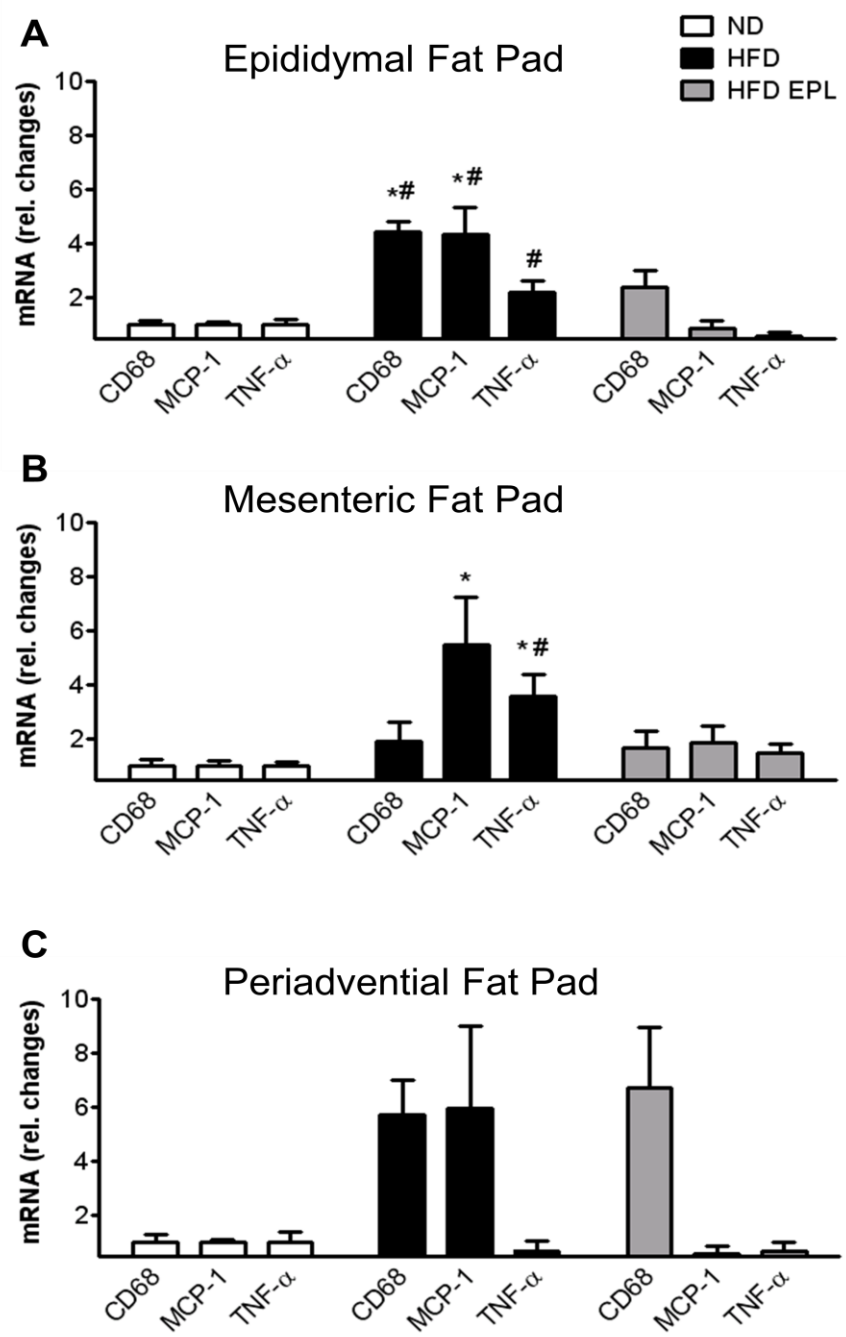


**Figure 24. Obesity-induced proinflammatory mRNA expression in white adipose tissue is attenuated by MR antagonism.** mRNA levels **(A)** epididymal fat pad, **(B)** mesenteric fat pad and **(C)** periadventitial fat pad in *C57BL/6* mice on ND, HFD and HFD EPL standardized to S12 and normalized to ND levels for CD68, MCP-1, and TNF-α. Data are mean±SEM; n=6–8.**P*<0.05 for ND vs HFD; #*P*<0.05 for HFD vs HFD EPL.

### 5.1.4 MR antagonism reverses obesity-induced endothelial dysfunction

The involvement of MR activation in the development of several cardiovascular diseases has been demonstrated in various studies[234,281-282]. Since endothelial dysfunction is an initial step in the development of cardiovascular diseases, we decided to investigate the effect of increased aldosterone levels in obesity on endothelial function. To do so, we performed organ chamber experiments using *C57BL/6* mice on ND, HFD and HFD EPL, respectively (Figure 25).

Endothelium-dependent relaxations to acetylcholine were blunted in aortae of obese mice starting at a concentration of $3x10^{-6}$ M acetylcholine and reaching a maximal relaxation at $10^{-4}$ M acetylcholine, proving pronounced endothelial dysfunction in obesity. Interestingly, chronic EPL treatment in obese mice restored endothelium-dependent relaxation completely back to levels of a ND (Figure 25A). Endothelium-independent relaxations, assessed by administration of the external NO donor sodium nitroprussid (SNP), were unaffected by obesity and reached a maximal relaxation in all 3 groups at $10^{-4}$ M SNP (Figure 25B). Furthermore, contractions to NE were unaffected by obesity and MR antagonism, respectively (Figure 25C).

These data suggest profound beneficial effects of MR antagonism on obesity-induced endothelial dysfunction.

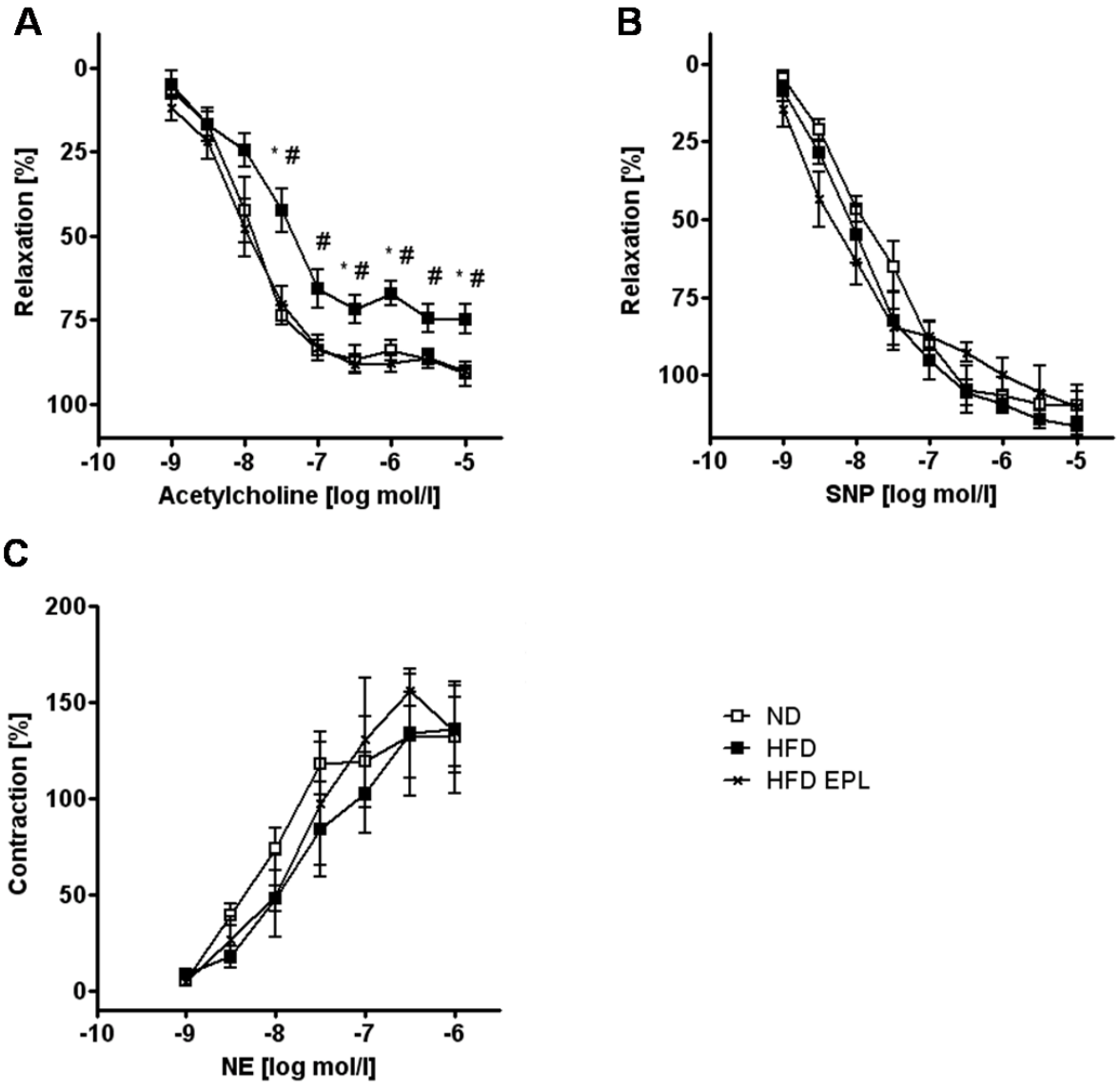


**Figure 25. MR antagonism restores obesity-induced endothelial dysfunction.** Endothelial function studies in *C57BL/6* mice on ND, HFD and HFD EPL. The response of aortic rings preconstricted with norepinephrine in increasing concentrations of (**A**) endothelium-dependent vasodilator acetylcholine (Ach) (**B**) and endothelium-independent vasodilator SNP. % Relaxation = % precontraction to norepinephrine. (**C**) Contraction of aortic rings at increasing norepinephrine concentrations. % Contraction = % of contraction to 80 mM KCl. Data are mean±SEM; n=8–10. **P*<0.05 for ND vs HFD; #*P*<0.05 for HFD vs HFD EPL.

### 5.1.5 MR antagonism diminishes obesity-induced expression of endothelial inflammation, prostanoid- and ROS-modulating enzymes

Since we could show that MR antagonism reverses obesity-induced endothelial dysfunction, we were wondering which mediators and signalling molecules were increased or downregulated in aortic endothelial cells, contributing to the improvement of endothelial function in obesity. Hence, we decided to develop a method to isolate fresh aortic endothelial cell mRNA from *C57BL/6* mice exposed to ND, HFD, HFD EPL (Materials and Methods; Figure 18) within 70 min after euthanasia of the mice.

In short, the aortic endothelial cell isolation procedure was divided into two main steps. The first one comprised the dissection and filling of the aorta with a 0.4% Collagenase/Dispase solution, followed by an incubation step for 30 min at 37°C to detach the endothelial cells from the vessel wall. The successful secession of aortic endothelial cells from the vessel lumen could be confirmed by immunofluorescence staining for the endothelial cell marker vWF. In a Collagenase/Dispase-untreated aorta, the vWF staining was clearly visible at the luminal endothelial cell layer (Figure 26A). Treatment of the aorta with the Collagenase/Dispase solution resulted in a negative vWF staining (Figure 26B), indicating the successful removal of endothelial cells.

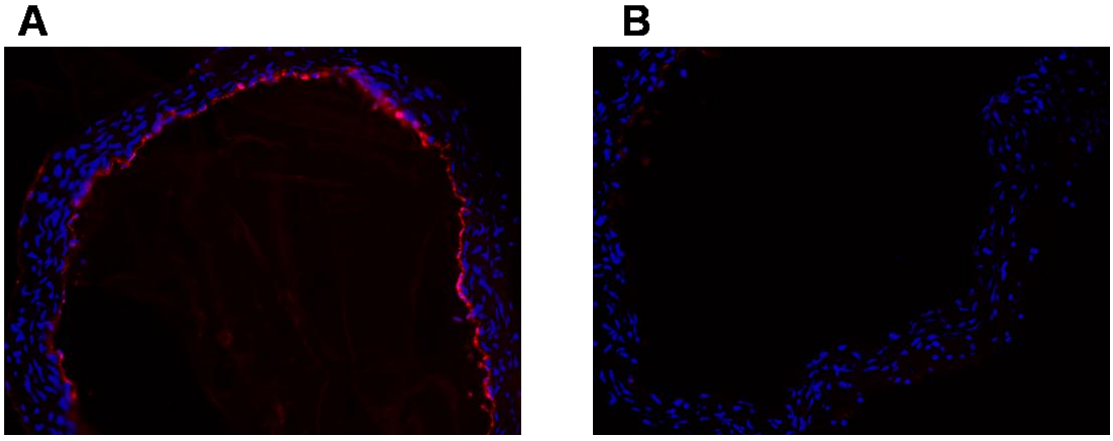


**Figure 26. Detachment of aortic endothelial cells from vessel wall after Collagenase/ Dispase treatment.** Immunofluorescence staining using a monoclonal anti-vWF antibody (1:400). (**A**) Collagenase/Dispase-untreated aorta and (**B**) Collagenase/Dispase-treated aorta.

The second main step in the aortic endothelial cell isolation procedure consisted of the purification of the isolated cells. Hence, the isolated cells were mixed with magnetic beads that were pre-coupled to an endothelial cell specific anti-CD31 antibody. After 20min of incubation, unbound cells were discarded, whereas all cells that bound to the beads-antibody-complex where supposed to be endothelial cells.

To confirm the purity of the aortic endothelial cells, the isolated cell fraction was stained for flow cytometry analyses. In turn, we used an isolectin B4-recognizing, biotinylated antibody that has been described to recognize murine endothelial cells, specifically[283]. We could show that the isolated cells express a relatively high amount of isolectin B4, suggesting that the isolated cells are indeed endothelial cells (Figure 27A). To verify this result, we compared the mRNA expression levels of the isolated cells with whole aortic lysate concerning endothelial cell marker vWF[284], smooth-muscle cell and fibroblast marker S100A4[285-286] and the hemoglobin, beta adult major chain (Hbb-b1) of erythrocyte (Figure 27B). The vWF mRNA was nearly 20-fold higher expressed in the isolated cell fraction in comparison to whole aortic tissue. In contrast, the markers of fibroblast and erythrocyte were low in isolated cells, thereby underlining the purity of our freshly isolated endothelial cells.

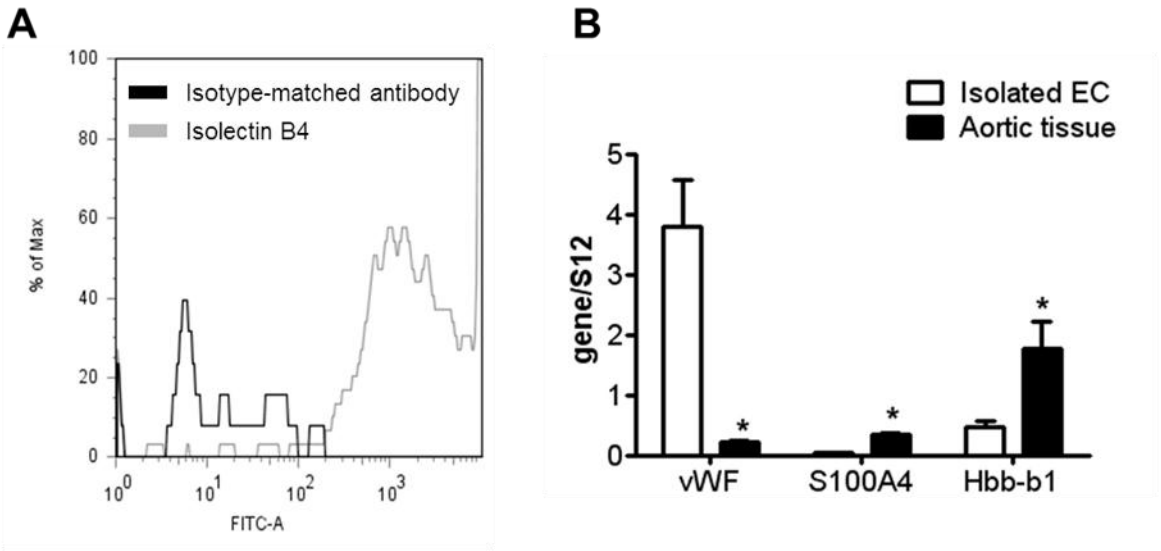


**Figure 27. Isolation of murine aortic endothelial cells.** (**A**) Flow cytometry analyses of aortic endothelial cells stained with biotinylated isolectin B4 as primary antibody and FITC-conjugated streptavidine as secondary antibody. (**B**) Aortic tissue and aortic endothelial cells isolated from *C57BL/6* mice. mRNA levels of endothelial cell marker vWF, fibroblast and smooth muscle cell marker S100A4, and erythrocyte marker Hbb-b1 standardized to S12. Data are mean±SEM; n=6–10. **P*<0.05 for isolated EC vs aortic tissue.

Since we could show, that we were able to isolate pure aortic endothelial cells from *C57BL/6* mice exposed to ND, HFD and HFD EPL, we tested the expression of proinflammatory molecules, thereby investigating the influence of both HFD and HFD EPL on mRNA expression patterns. ICAM-1 mRNA showed a non-significant increase with obesity and a marked decrease by eplerenone in obese mice; VCAM-1 expression increased upon HFD exposure but remained unaltered with eplerenone. Moreover, expression of the C1-inhibitor and MR was induced by eplerenone in obesity (Figure 28A). Interestingly, we could observe a slight increase of MR mRNA expression under HFD and an even higher increase upon EPL treatment (Figure 28A); the same MR mRNA pattern as observed in whole aortic tissue and WAT (Figure 23).

A closer look on mediators involved in the development of endothelial dysfunction revealed that prostacyclin synthase and COX-1 mRNA were upregulated in obesity, whereas COX-2 expression was unaltered (Figure 28B). Furthermore, eNOS mRNA expression increased upon HFD EPL exposure compared with the ND (Figure 28B).

Taken together, we observed an obesity-induced expression of proinflammatory mediators in obesity that was partially attenuated by MR antagonism. In addition, we could show that obesity enhances the expression of enzymes that are involved in the generation of vasoconstricting prostanoids and that MR antagonism increases the expression of eNOS in obesity.

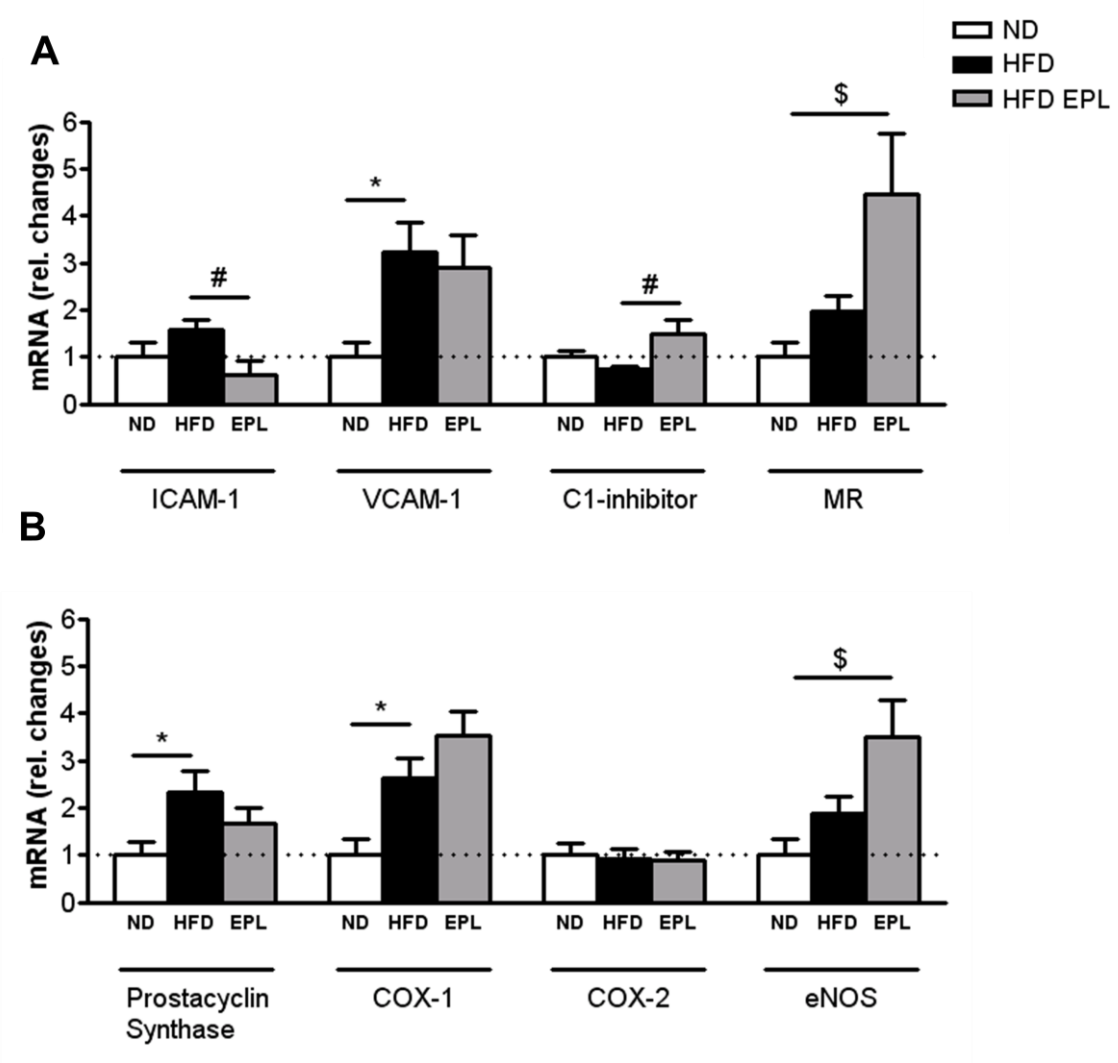


**Figure 28. Some obesity-induced proinflammatory and prostanoid-related changes are alterd by MR antagonism in aortic endothelial cells.** Aortic endothelial cell mRNA levels in *C57BL/6* mice on ND, HFD, HFD EPL (EPL) after 14 weeks standardized to S12 (**A**) ICAM-1, VCAM-1, C1-inhibitor, MR, (**B**) prostacyclin-synthase, COX-1, COX-2, eNOS. Data are mean±SEM; n=9-12. **P*<0.05 for ND vs HFD; #*P*<0.05 for HFD vs HFD EPL; $*P*<0.05 for ND vs HFD EPL.

Given the link of aldosterone to oxidative stress[251,254,257], we decided to analyze the isolated aortic endothelial cell mRNA from ND, HFD and HFD EPL fed mice concerning expression levels of antioxidant systems and NADPH-oxidase subunits. Regarding antioxidant systems, we found levels of SOD-1 and SOD-3 increased under ND and HFD EPL conditions in comparison to HFD; catalase expression was not altered significantly (Figure 29A). In addition, we found that mRNA expression of the NADPH-oxidase subunits p22phox, p47phox, and gp91phox was not affected by obesity. However, EPL treatment significantly attenuated the expression of p22phox and p47phox (Figure 29B). On the other hand, obesity increased endothelial expression of p40phox and Rac-1 that was attenuated by EPL (Figure 29B). Taken together, we observed an obesity-

induced oxidative stress-associated mRNA expression profile in aortic endothelial cells that was partially restored by MR antagonism.

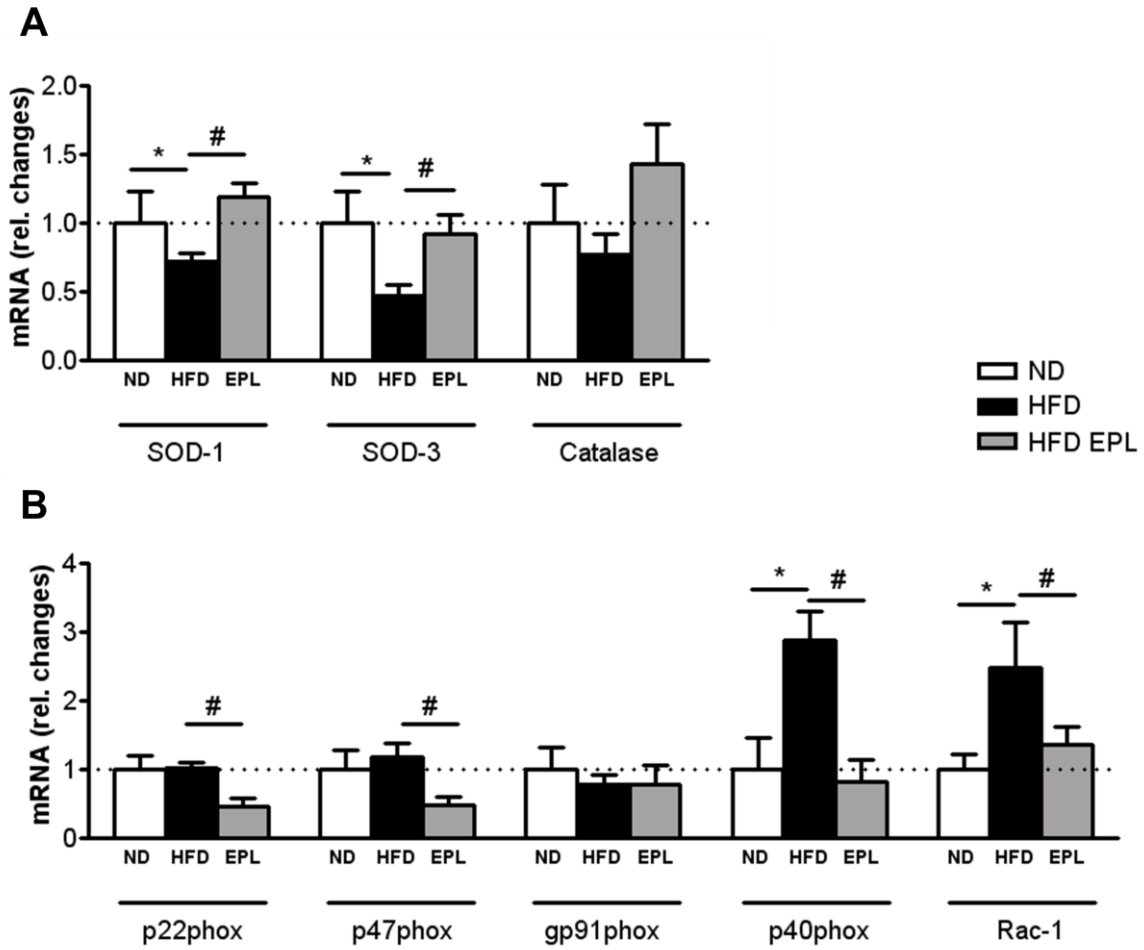


**Figure 29. Obesity-induced oxidative stress is attenuated by MR antagonism in aortic endothelial cells.** Aortic endothelial cell mRNA levels in *C57BL/6*mice on ND, HFD, HFD EPL (EPL) after 14 weeks standardized to S12 (**A**) SOD-1, SOD-3, catalase,(**B**) p22phox, p47phox, gp91phox, p40phox, Rac-1. Data are mean±SEM; n=9-12. **P*<0.05 for ND vs HFD; #*P*<0.05 for HFD vs HFD EPL.

Together with the increased expression of inflammatory markers and prostanoid-generating enzymes, the enhanced expression of endothelial oxidative stress-related systems might contribute to the development of endothelial dysfunction in obesity. MR antagonism seems to attenuate this protective EC expression profile, being therefore responsible for improved endothelial function in obesity.

## 5.2 Endothelial MR ablation prevents obesity-induced endothelial dysfunction

### 5.2.1 Ablation of the endothelial MR – generating EC MR KO mice

In the previous section we were able to show a beneficial effect of MR antagonism on obesity induced-endothelial dysfunction as well as on inflammatory, prostanoid- and oxidative stress-related endothelial cell mRNA expression pattern.

In order to characterize the obesity-induced changes in the endothelium in more detail, we generated mice lacking the MR in endothelial cells (*EC MR KO)* by crossing floxed *MR* mice with *Tie2-Cre* mice. To verify the successful action of the CreLoxP system in this context, we performed qPCR analyses of aortic endothelial cell DNA, isolated from *MR WT* and *EC MR KO* mice, checking the successful excision of the MR. Indeed, we could show that the MR knock out appeared to be 92.8% (±3.2%) efficient in *EC MR KO* mice in comparison to their *MR WT* littermates (Figure 30A).

In order to determine the specificity of the EC MR KO, we assessed MR expression the whole aortic tissue, peritoneal macrophages and epididymal WAT (Figure 30B). Whereas MR expression was almost unaltered in whole aortic tissue and WAT, the MR seems to be ablated in peritoneal macrophages. This phenomenon has been already reported previously in hematopoietic cells[287] and is due to an unspecific activation of the Tie2 promoter in these cells.

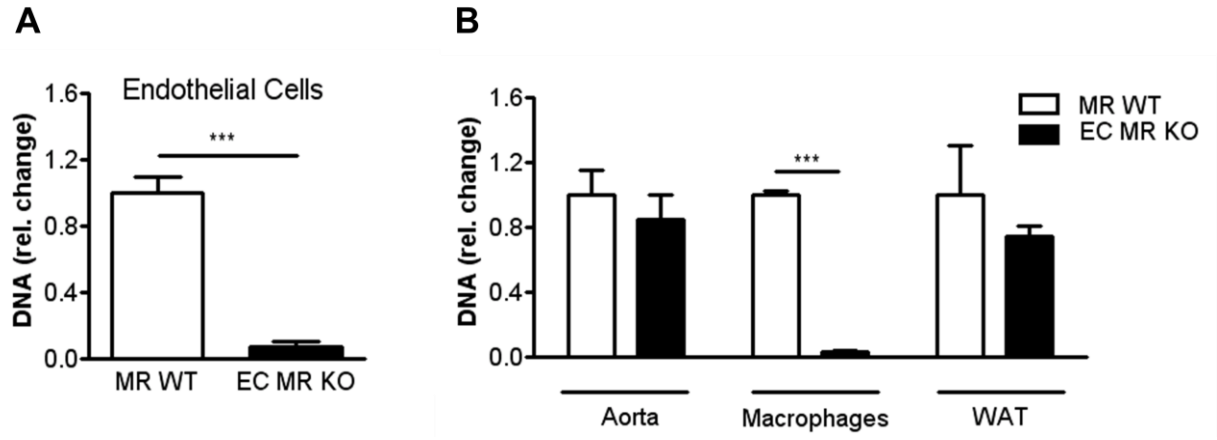


**Figure 30. MR ablation in *EC MR KO* mice.** MR DNA levels of (**A**) endothelial cell (n=8), (**B**) peritoneal macrophages, epididymal fat pad and whole aortic lysates (n=6) in *MR WT* and *EC MR KO* mice standardized to S12, **P<0.05*. Values are mean ± SEM.

### 5.2.2 Endothelial MR ablation has no effect on obesity-associated glucose intolerance

To elucidate the role of the endothelial MR in diet-induced obesity, we exposed *EC MR KO* mice and their corresponding *MR WT* littermates to a ND or HFD for 14 weeks. Relative weight gain and epididymal fat mass did not differ between *EC MR KO* and *MR WT* animals neither under ND nor under HFD conditions (Figure 31A, B).

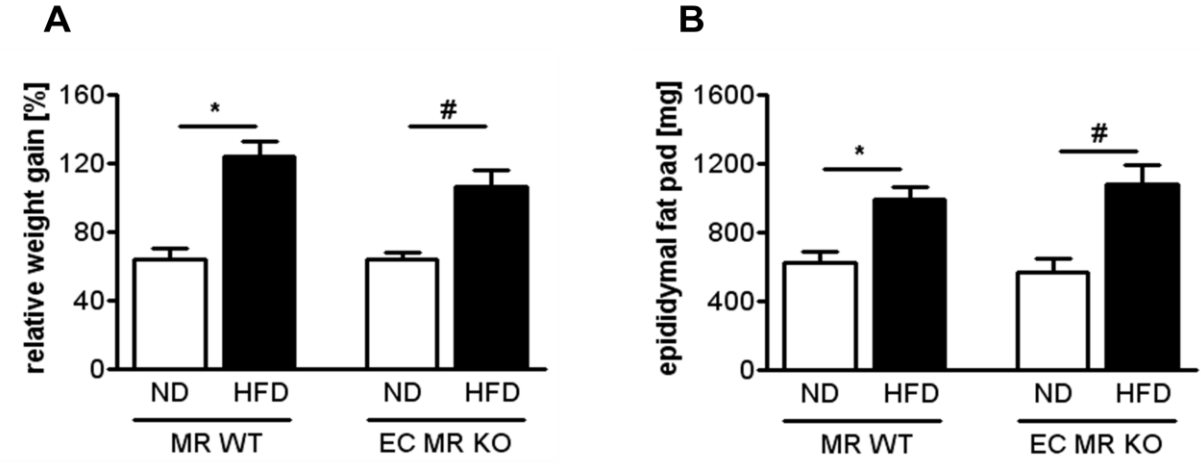


**Figure 31. Similar high-fat diet induced increase in weight gain in lean and diet-induced obese *MR WT* and *EC MR KO* mice.** *MR WT* and *EC MR KO* mice were treated with ND and HFD for 14 weeks. After 14 weeks (**A**) relative weight gain and (**B**) epididymal fat pad mass were measured. Data are mean±SEM; n=6–8.*$P$<0.05 for *MR WT* ND vs *MR WT* HFD; #$P$<0.05 for *EC MR KO* ND vs *EC MR KO* HFD.

To check whether endothelial cell MR ablation affects obesity-induced hyperglycemia, we tested blood glucose of *MR WT* and *EC MR KO* mice after 12 weeks of ND or HFD. We found no difference in fed and fasted blood glucose in *MR WT* and *EC MR KO* mice, neither under ND nor under HFD (Figure 32A, B). Glucose tolerance tests revealed an impaired glucose tolerance in both obese *MR WT* and *EC MR KO,* respectively (Figure 32C). Since glucose tolerance seemed to be impaired to the same extent in *MR WT* and *EC MR KO*, we concluded that EC MR plays no role in obesity-associated diabetes.

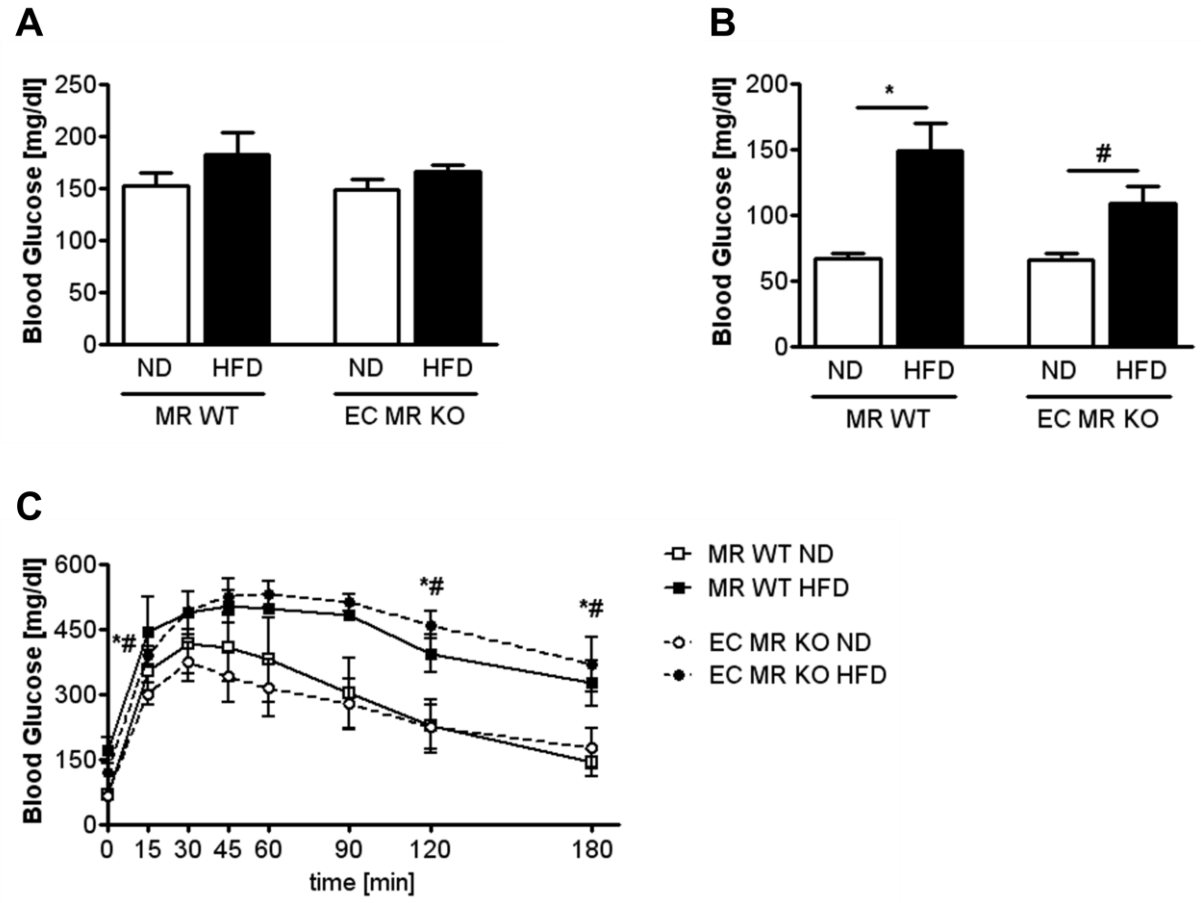


**Figure 32. Glucose tolerance in lean and diet-induced obese *MR WT* and *EC MR KO* mice.** *MR WT* and *ECMR KO* mice were treated with ND and HFD for 14 weeks. After 12 weeks of HFD, blood glucose was determined (**A**) under fed state (**B**) after fasting for 16 h over night and (**C**) in a time course after injection of 2 g glucose/kg body weight. Data are mean±SEM; n=6–8.&*P*<0.05 for *MR WT* HFD vs *MR WT* ND; #*P*<0.05 for *EC MR KO* ND vs *EC MR KO* HFD.

### 5.2.3 Endothelial MR ablation has no effect on obesity-induced increase of plasma aldosterone

To test whether endothelial MR deletion affects aldosterone generation or degradation, we measured aldosterone concentration in the plasma of *MR WT* mice and *EC MR KO* after 14 weeks of ND or HFD. Both obese *MR WT* mice and *EC MR KO* showed a similar increase in plasma aldosterone (Figure 33A). Therefore, endothelial MR has no relevant effect on aldosterone turnover.

MR mRNA analysis of whole aortic tissue isolated from *MR WT* mice and *EC MR KO* revealed that the MR mRNA expression in wild-type and KO mice is similar in the whole vessel. Furthermore, obesity did not enhance MR mRNA expression in *MR WT* and *EC MR KO*, respectively (Figure 33B). We observed a similar MR mRNA expression pattern in the aorta and in the epididymal WAT (Figure 33C).

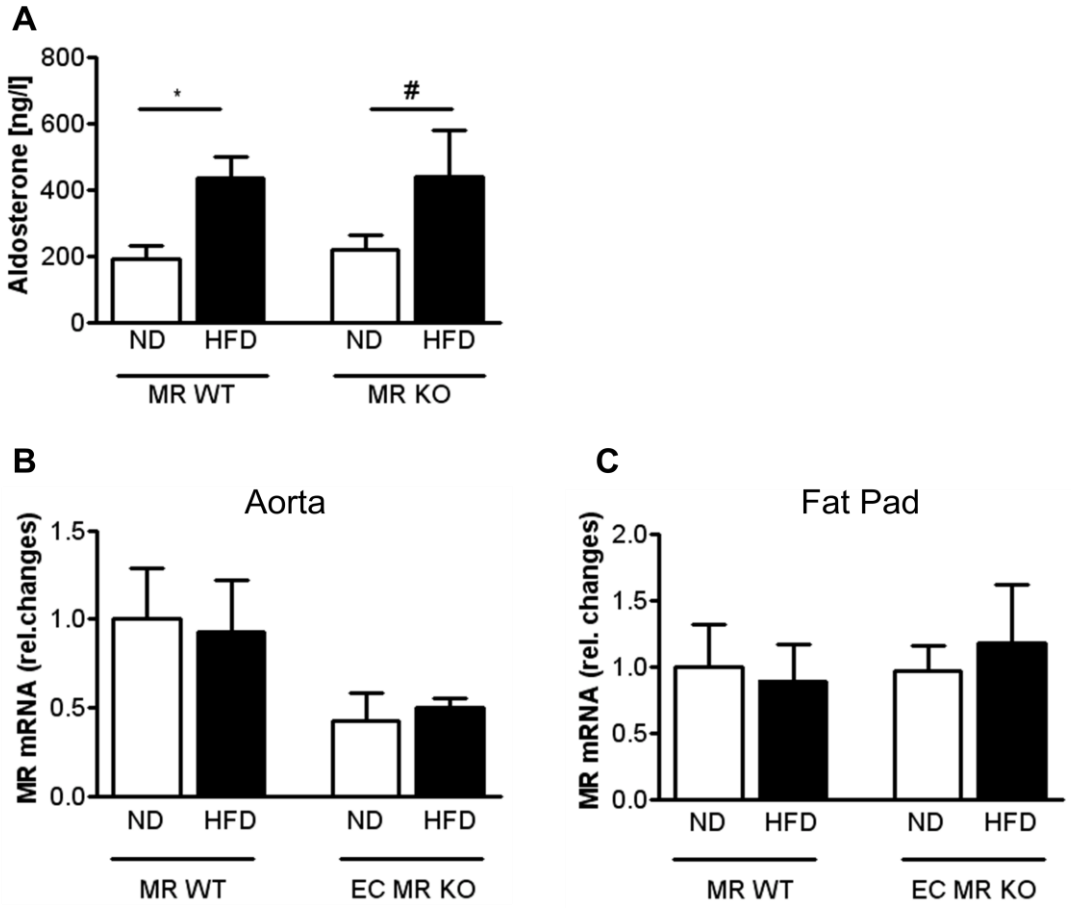


**Figure 33. Plasma aldosterone levels in lean and diet-induced obese *MR WT* and *EC MR KO* mice.** *MR WT* and *EC MR KO* mice on ND or HFD were analyzed concerning **(A)** plasma aldosterone levels **(B)** MR mRNA of aorta **(C)** epididymal fat pads, standardized to S12 and normalized to ND. Data are mean±SEM; n=6-8.*$P$<0.05 for *MR WT* ND vs *MR WT* HFD; #$P$<0.05 for *EC MR KO* ND vs *EC MR KO* HFD.

### 5.2.4 Endothelial MR ablation has no effect on obesity-induced proinflammatory changes in the WAT

In a next step, we wanted to test the effect of the endothelial MR deletion on proinflammatory cytokine expression in the WAT. In previous experiments of diet-induced obesity we observed the most pronounced anti-inflammatory effects by MR blockade in the epididymal fat pad (Figure 6A). Therefore, we decided to test, if an endothelial MR deletion is sufficient to reverse proinflammatory changes in the epididymal WAT in obesity.

We exposed *MR WT* and *EC MR KO* mice to a ND or HFD for 14 weeks and analyzed the epididymal WAT concerning the expression of proinflammatory markers. TNF-α, MCP-1 and the macrophage marker CD68 mRNA levels increased significantly to the same extent in *MR WT* and *EC MR KO* mice upon HFD exposure (Figure 34). These data suggest that endothelial MR deletion has no effect on the pro-inflammatory state of the WAT. Moreover, these findings imply that the MR deletion in macrophages of the WAT has no relevant effect on the inflammatory profile of the WAT.

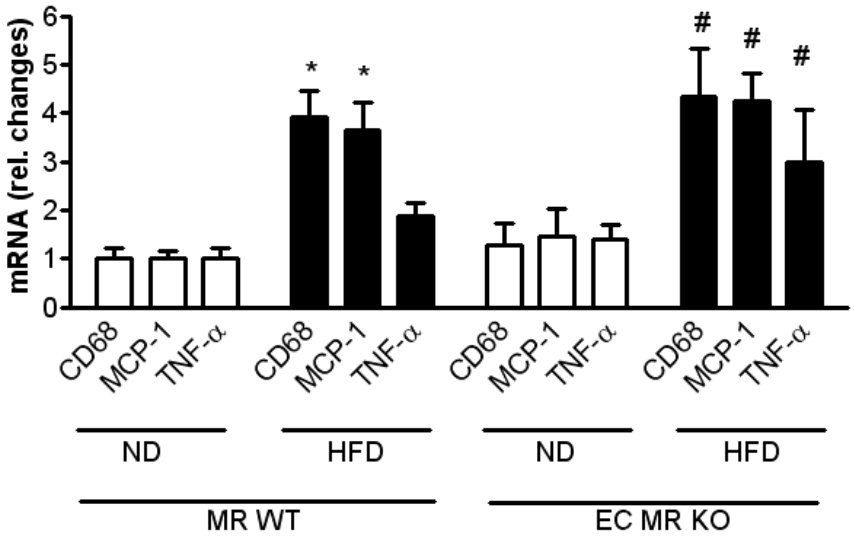


**Figure 34. Obesity-induced and aldosterone-induced proinflammatory changes in white adipose tissue are unaltered by endothelial MR KO.** mRNA levels of epididymal fat pad in *MR WT* and *EC MR KO* mice on ND or HFD were standardized to S12 and normalized to ND levels for CD68, TNF-α, MCP-1. Data are mean±SEM; n=6-8.*$P$<0.05 for *MR WT* ND vs *MR WT* HFD; #$P$<0.05 for *EC MR KO* ND vs *EC MR KO* HFD.

### 5.2.5. Endothelial MR ablation prevents obesity-induced impairment of vasodilation

To determine whether obesity-induced endothelial dysfunction was mediated by the endothelial MR, we exposed *WT* and *EC MR KO* mice to a ND or HFD for 14 weeks and performed organ chamber experiments. Compared with lean controls, obese *MR WT* mice demonstrated a significant impairment of endothelium-dependent vasodilation starting at $10^{-7}$ M acetylcholine; strikingly, we could show that a MR deletion in endothelial cells completely abolished the endothelial dysfunction (Figure 35A). Endothelial-independent SNP-induced relaxation (Figure 35B) and contractions induced by norepinephrine (Figure 35C) were similar in *MR WT* and *EC MR KO* on ND or HFD, respectively.

Therefore, we conclude that the endothelial MR plays a crucial role in the development of obesity-induced endothelial dysfunction.

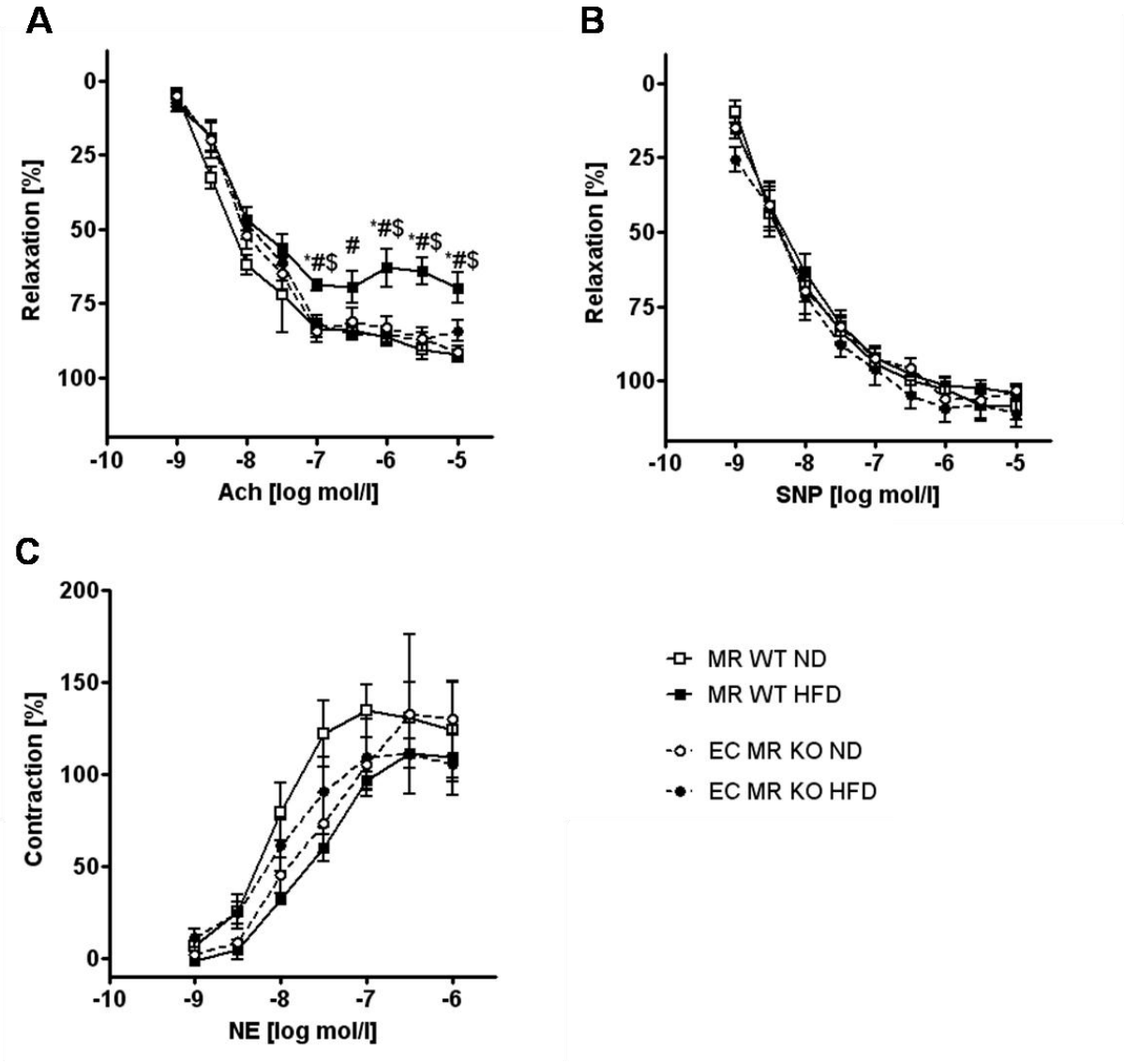


**Figure 35. Obesity-induced endothelial dysfunction is prevented in *EC MR KO* mice.** Endothelial function studies in *MR WT* and *EC MR KO* mice on ND and HFD: The response of aortic rings preconstricted with norepinephrine to increasing concentrations of (**A**) endothelium-dependent vasodilator acetylcholine (Ach) (**B**) and endothelium-independent vasodilator SNP. % Relaxation = % precontraction to norepinephrine. (**C**) Contraction of aortic rings at increasing norepinephrine concentrations. % Contraction = % of contraction to 80 mM KCl. Data are mean±SEM; n=6–8. **P*<0.05 for *MR WT* ND vs *MR WT* HFD; #*P*<0.05 for *MR WT* HFD vs *EC MR KO* HFD; $*P*<0.05 for *MR WT* HFD vs *EC MR KO* ND.

## 5.3 Endothelial MR ablation attenuates aldosterone-induced endothelial dysfunction by modulating enzymes, associated with prostanoid and ROS production

Subsequently, we tested whether the effects in obesity associated with high endogenous aldosterone could be mimicked with high exogenous aldosterone alone without obesity.

To do so, we implanted osmotic minipumps in lean MR WT mice and EC MR KO mice containing aldosterone or vehicle. Aldosterone infusion for 2 weeks increased plasma aldosterone to the same extent in both *MR WT* and *EC MR KO* mice (Figure 36). Since we were able to obtain a similar increase of plasma aldosterone by exogenous administration as obesity induces aldosterone production endogenously, we could dissect the role of increased aldosterone without the additional metabolic and inflammatory changes conferred by obesity.

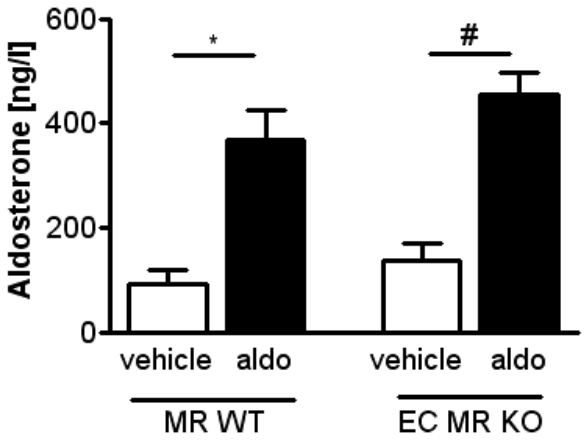


**Figure 36. Plasma aldosterone levels in vehicle- or aldosterone-infused lean *MR WT* and *EC MR KO* mice.** *MR WT* and *EC MR KO* mice on ND or HFD and vehicle-, or aldosterone-infused mice were analyzed concerning plasma aldosterone levels. Data are mean±SEM; n=6-8.*$P$<0.05 for *MR WT* vehicle vs *MR WT* aldo; #$P$<0.05 for *EC MR KO* vehicle vs *EC MR KO* aldo.

### 5.3.1 Endothelial MR ablation and COX inhibition restore aldosterone-induced impaired vasodilation

To determine whether obesity-induced, endothelial MR-mediated endothelial dysfunction was induced by aldosterone, MR-dependent or independent pathways we tested endothelial function of aldosterone- or vehicle-infused *MR WT* and *EC MR KO* mice. Infusion of aldosterone for 2 weeks blunted endothelial function in concentrations of acetylcholine above $10^{-7}$mol/l. Indeed, deletion of the endothelial MR attenuates aldosterone-induced endothelial dysfunction, completely (Figure 37A). Endothelium-independent relaxation by SNP (Figure 19B) and contractions induced by norepinephrine (Figure 37C) were similar in all groups.

Since we observed increased mRNA expression of COX-1 and prostaglandin synthase in aortic endothelial cells in obesity (Figure 28B), we tested the effects of a COX-1/COX-2 inhibition on exogenous aldosterone-induced endothelial dysfunction. We could clearly show that inhibition of COX by indometacin normalizes endothelial function in aldosterone-infused mice to the same extent as endothelial MR deletion (Figure 37D).

We conclude from these results, that the endothelial MR plays a crucial role in obesity- as well as in aldosterone-induced endothelial dysfunction. Since we could show that COX seems to contribute to aldosterone induced-endothelial dysfunction, we suggest that the endothelial MR induces COX-1 expression.

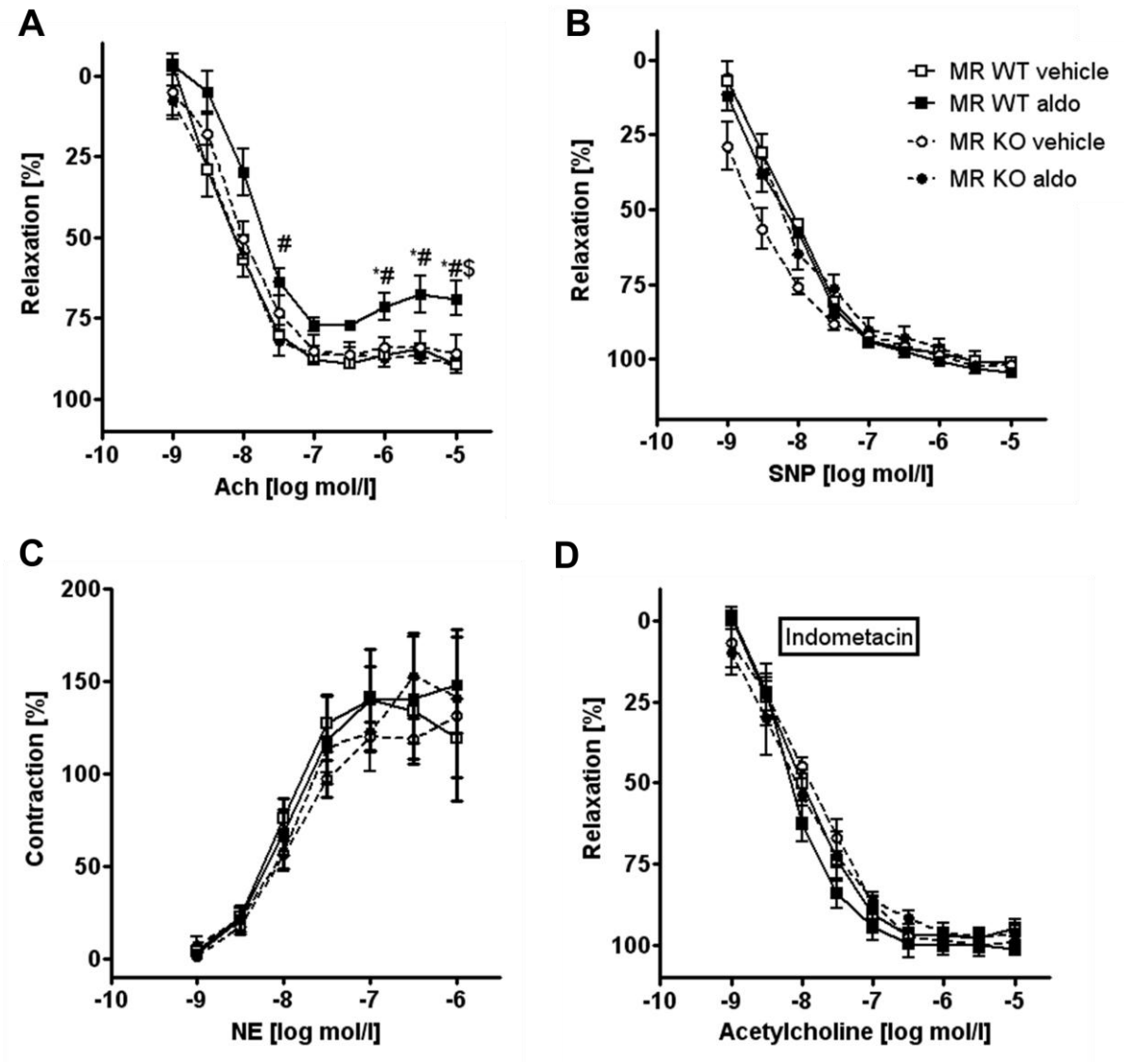


**Figure 37. Aldosterone-induced endothelial dysfunction is prevented in lean *EC MR KO* mice and upon COX inhibition, respectively.** Endothelial function studies in *MR WT* and *EC MR KO* mice after aldosterone or vehicle infusion: The response of aortic rings preconstricted with norepinephrine to increasing concentrations of (**A**) endothelium-dependent vasodilator acetylcholine (Ach), (**B**) endothelium-dependent vasodilator Ach and preincubated with indometacine and (**C**) endothelium-independent vasodilator SNP. % Relaxation = % precontraction to norepinephrine. (**D**) Contraction of aortic rings at increasing norepinephrine concentrations. % Contraction = % of contraction to 80 mM KCl. Data are mean±SEM; n=6–8. **P*<0.05for*MR WT* ND vs *MR WT* HFD; #*P*<0.05 for *MR WT* HFD vs *ECMR KO* HFD; $*P*<0.05 for *MR WT* HFD vs *EC MR KO* ND.

### 5.3.2 Exogenous aldosterone infusion does not induce the expression of proinflammatory markers in the WAT

To test the effects of endothelial MR ablation on aldosterone-induced inflammation in the WAT, we infused *MR WT* and *EC MR KO* mice with aldosterone for 2 weeks and tested expression proinflammatory markers in the epididymal WAT. Aldosterone infusion did not increase mRNA levels of TNF-α, MCP-1 or CD68 neither in *MR WT* nor in *EC MR KO* mice (Figure 38). These data suggest that increased aldosterone in obesity is not involved in inducing proinflammatory changes in the adipose tissue. Furthermore, we conclude that an endothelial MR deletion has no effect on the pro-inflammatory state of the WAT.

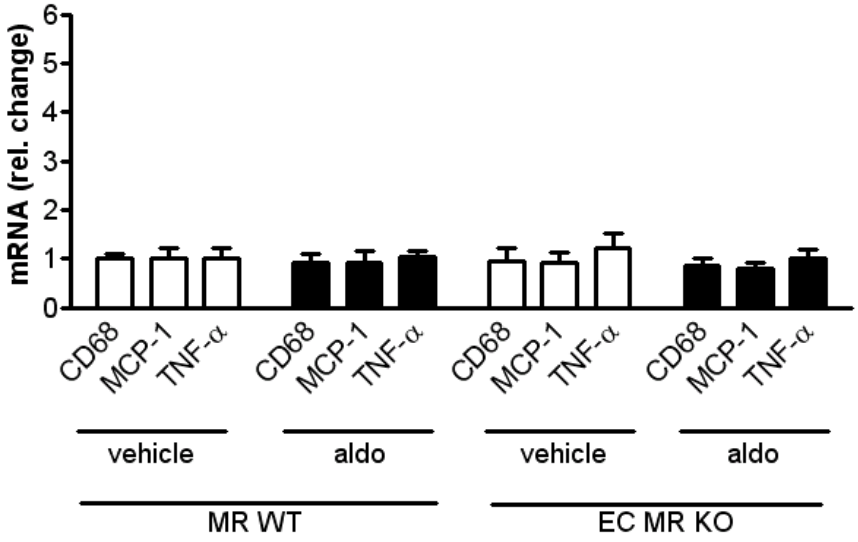


**Figure 38. The expression level of proinflammatory gene products is not altered in the WAT by aldosterone and/or endothelial MR ablation.** mRNA levels of epididymal fat pad in vehicle- or aldosterone-infused *MR WT* and *EC MR KO* mice were standardized to S12 and normalized to ND levels for CD68, TNF-α, MCP-1. Data are mean±SEM; n=6-8.**P*<0.05 for *MR WT* ND vs *MR WT* HFD; #*P*<0.05 for *EC MR KO* ND vs *EC MR KO* HFD.

### 5.3.3 Endothelial MR ablation diminishes aldosterone-induced expression of endothelial prostanoids- and ROS-modulating enzymes

In a next step, we investigated which obesity-induced pro-inflammatory, vasoconstricting or pro-oxidative mediators were directly enhanced by aldosterone or the endothelial MR in aortic endothelial cells. To do so, we isolated fresh aortic endothelial cells from *MR WT* and *EC MR KO* mice, infused with aldosterone for 2 weeks.

Concerning inflammatory changes, we could not detect ICAM-1 and VCAM-1 mRNA expression by qPCR measurements (data not shown); C1-inhibitor mRNA was expressed, but unaltered in all conditions (Figure 39A). Interestingly, we observed a decrease in MR mRNA in *EC MR KO* mice, suggesting a successful ablation of the MR in aortic endothelial cells (Figure 39A). Testing the expression of mediators that are involved in the development of endothelial dysfunction showed that prostacyclin synthase mRNA expression is significantly enhanced by exogenous aldosterone, independent of the endothelial MR (Figure 39B). On the other hand, aldosterone-induced COX-1 mRNA expression was significantly decreased in *EC MR KO* mice (Figure 39B). COX-2 and eNOS mRNA expression were not significantly altered by aldosterone or endothelial MR KO (Figure 39B).

Taken together, exogenous aldosterone alone did not induce expression of proinflammatory mediators in aortic endothelial cells. In contrast, aldosterone seems to induce prostacyclin expression in an endothelial MR-independent manner and influence COX-1 expression in an endothelial MR-dependent manner.

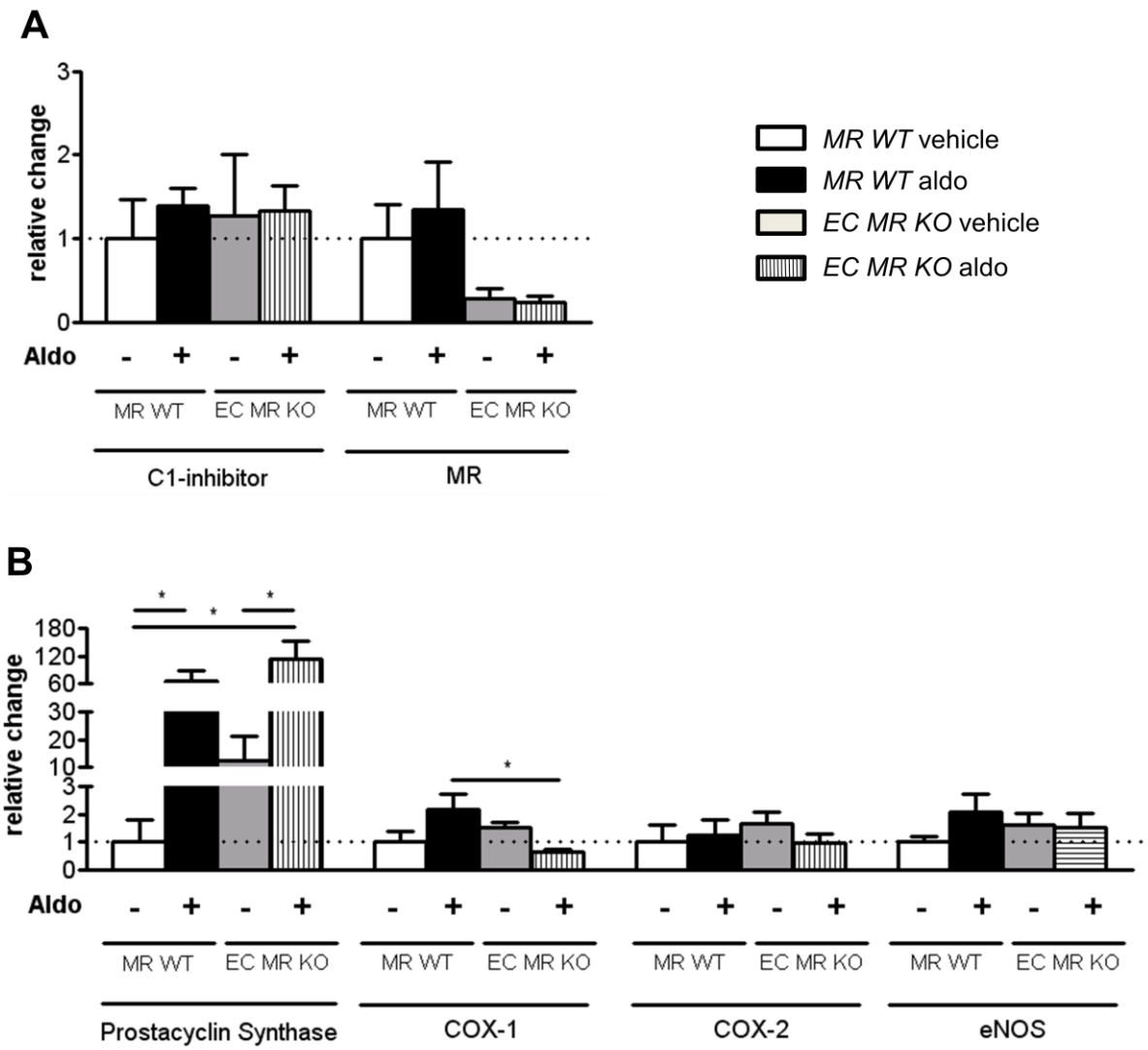


**Figure 39. Aldosterone-induced changes are partially attenuated by MR antagonism in aortic endothelial cells.** Aortic endothelial cell mRNA levels in *MR WT* and *EC MR KO* mice vehicle-, or aldosterone-infused for 2 weeks standardized to S12 (**A)** C1-inhibitor, MR, (**B**) prostacyclin-synthase, COX-1, COX-2, eNOS. Data are mean±SEM; n=5-7. **P*<0.05.

Investigations concerning enzyme systems involved in modulating oxidative stress revealed that mRNA expression of the antioxidant system SOD-1 inclined by aldosterone. In *EC MR KO* mice SOD-1 expression seemed to be increased again, in presence and absence of aldosterone. Moreover, SOD-3 mRNA was significantly decreased by aldosterone treatment. Interestingly, SOD-3 mRNA was also found to be attenuated in *EC MR KO* mice in the presence or absence of aldosterone (Figure 40A).

Regarding the NADPH oxidase subunits, we found the p22phox mRNA is significantly upregulated by aldosterone. However, in *EC MR KO* mice, the expression of the subunit was abolished again (Figure 40B). Whereas gp91phox was unaltered in all conditions (Figure 40B), the mRNAs of the other NADPH oxidase subunits, namely p47phox, p40phox and Rac-1, as well as the antioxidant system catalase could not be detected by qPCR (data not shown).

Taken together, aldosterone seems to alter the expression of mediators involved in the generation of ROS and prostanoids in an endothelial MR-dependent manner. By doing this, aldosterone contributes to the development of endothelial dysfunction, at least in part, via MR signaling.

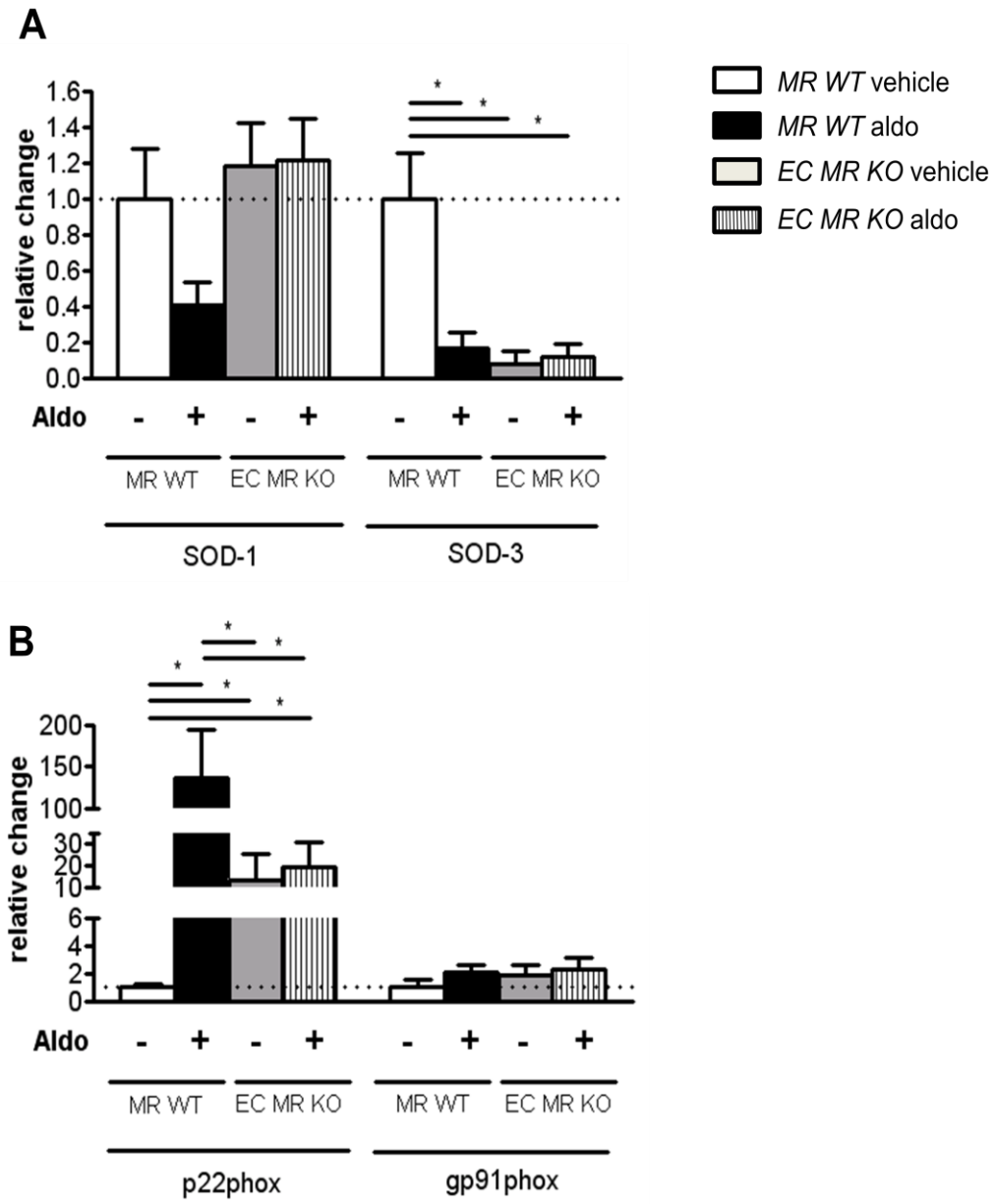


**Figure 40. Aldosterone-induced oxidative stress-related changes are attenuated by MR antagonism in aortic endothelial cells.** Aortic endothelial cell mRNA levels in *MR WT* and *EC MR KO* mice vehicle- or aldosterone-infused for 2 weeks standardized to S12 (**A**) SOD-1, SOD-3, (**B**) p22phox, gp91phox. Data are mean±SEM; n=5-7. **P*<0.05.

# 6 Discussion

The data obtained in this PhD thesis contribute to the understanding of the role of aldosterone in obesity-induced endothelial dysfunction and evaluate for the first time *in vivo* the role of the endothelial MR in this context. Our experimental study provided three major findings:

- Diet-induced obesity increases proinflammatory cytokines in the WAT and enhances expression of mediators involved in generation of oxidative stress as well as in generation of vasoconstricting prostanoids in aortic endothelial cells. Thereby, obesity induces endothelial dysfunction.

- Pharmacological inhibition of the MR diminishes these changes. It attenuates proinflammatory cytokine expression in the WAT, tips the balance of pro-oxidative parameters in aortic endothelial cells towards the anti-oxidative side and induced the expression of eNOS. These beneficial changes reverse obesity-induced endothelial dysfunction.

- Deletion of endothelial MR is sufficient to protect from harmful effects induced by obesity or exogenous aldosterone in the aorta: Endothelial MR deletion diminishes aldosterone-induced effects in aortic endothelial cells, tips expression of ROS-regulating enzymes towards endothelial protection and abolishes thereby aldosterone-induced endothelial dysfunction.

- Prostanoids appear to play a dominant role in obesity and aldosterone-induced endothelial dysfunction based on the strong impact HFD, aldosterone infusion and endothelial MR knock-out have on endothelial COX-1 and prostacyclin synthase mRNA expression and the fact that COX-inhibition by indomethacin reverts aldosterone-induced endothelial dysfunction.

# 6.1 Pharmacological MR antagonism in diet-induced obese mice

## 6.1.1 Metabolic changes

In order to characterize the effects of endogenous aldosterone in obesity on the development of endothelial dysfunction, we analyzed metabolic, inflammatory and vascular changes in diet-induced obese mice. We observed an increase in relative body weight gain in all obese mice, independent of MR antagonism. Therefore, we suggest that MR signaling is not affecting body weight. These findings are in line with a recent report investigating the effects of MR antagonism in genetically obese mice[219].

In addition to that, we showed that pharmacological blockade of MR in obese mice attenuates hyperglycemia and improves glucose tolerance. Indeed, MR antagonism has been reported to reduce insulin resistance in genetically obese mice[216,219]. Aldosterone impairs insulin-induced glucose uptake by two ways: first by decreasing the expression of glucose transports in the skeletal muscle and in the liver[224] and second, by degrading the IRS-1 and -2 in cultured adipocytes[225]. These effects may account for skeletal, hepatic and adipocyte insulin resistance in obesity and diabetes.

## 6.1.2. RAAS-related changes

We demonstrated that plasma aldosterone concentration is increased in diet-induced obese mice. This observation has been also been reported previously[200-201] but it remains unclear why aldosterone is increased during weight gain. Obesity-induced systemic RAAS activation seems unlikely, since we and others[202,288] observed no change in renin plasma concentration or activity in obesity. Activation of a local adipose RAAS, contributing to the increased aldosterone production, can be ruled out, since adipocytes are not expressing aldosterone synthase[208]. In addition, adipose tissue derived- AngII is unable to enhance aldosterone secretion in adrenocortical cells[208].

RAS-independent factors have been demonstrated to stimulate aldosterone secretion in obesity. The oxidized derivative of linoleic acid EKODE, which is increased in obesity, stimulates aldosterone secretion in adrenocortical cells directly[206-207]. Moreover, the increased release of so far unidentified aldosterone-releasing factors by the WAT in obesity stimulates aldosterone production[209]. Interestingly, classical adipokines such as IL-6, TNF-α, and leptin can be excluded as direct stimuli of adrenal steroidogensis[208]. This suggests that not the systemic or local adipose RAS induces the aldosterone production in obesity, but rather the WAT itself, in its function as fat storage organ and as endocrine organ, plays a crucial role in the increased aldosterogenesis.

The increased aldosterone levels in obesity might play an important role in inducing endothelial dysfunction and inflammation in this context.

Besides from the elevated plasma aldosterone levels in obesity, we could show that increase in aldosterone is even more pronounced when EPL is administered. This is in line with a previous publication showing that MR antagonism increases plasma aldosterone levels in genetically obese mice[219]. The increase in plasma aldosterone after EPL treatment implies that the blockade of MR is efficient and, due to a missing negative feedback loop, enhances aldosterone release.

Surprisingly, we did not observe an increased urinary aldosterone level, in high fat diet-obese mice, unlike shown by others[219] in genetically obese (*db/db*) mice. There might be several explanations for this observation. One could be that aldosterone degrading reductases might be more active in obese mice leading to shorter half-life of aldosterone in the circulation. This possibility is however not likely in view of the lack of increase in urinary corticosterone metabolites shown in Figure 22B (same reductases). Another explanation might be that obesity blunts the circardian variations of aldosterone production and favours a rather continuous aldosterone level. This would explain the relatively high aldosterone levels measured in the plasma of high fat diet-obese mice at a time, when endogenous aldosterone is very low in mice maintained under normal diet (mice were sacrificed 4h after beginning of the light period) and the rather low 24h averaged urine aldosterone levels.

Apart from increased plasma aldosterone levels in obesity, we observed a non-significant increase in MR mRNA expression in aortae and WAT of obese *C57BL/6*. So far, only one study by Hirata[216] shows an increased MR mRNA expression in adipocytes from genetically obese mice. They propose that enhanced MR expression is due to increased ROS levels in the WAT[216] and suggest that the emerging oxidative stress might induce the expression of adipose MR that further augments fat ROS production and inflammation of adipose tissue. Since we do not observe a significant MR regulation in the adipose tissue of obesity, we rather suggest that aldosterone and not oxidative stress is responsible for MR activation in our mouse model of diet-induced obesity.

### 6.1.3 White adipose tissue inflammation

Obesity alters genes expression and induces inflammation in the WAT of genetically obese mice[289] similar to the changes reported in humans[55,290]. Along that line, we observed an increased expression of MCP-1, TNF-α and macrophage marker CD68 in the obese epididymal, mesenteric and periadventitial WAT.

This study as well as others[216,219] could show that EPL decreases mRNA of obesity- and inflammation-related changes such as MCP-1 and TNF-α mRNA expression as well as macrophage infiltration in the epididymal, mesenteric and periadventitial WAT, suggesting that obesity is associated with an activated MR. Indeed, the MR has been shown to regulate transcription through inhibition of AP-1/NFκB protein–DNA complexes[291] that can influence the expression of proinflammatory adipokines like TNF-α, IL-1β, IL-6 and leptin[219,292-294]. Since obesity-associated proinflammatory changes in the WAT such as TNF-α and MCP-1 can contribute to the development of insulin resistance[27,295], we propose that decreased expression of adipokines by MR antagonism participates in the improved glucose tolerance in our mouse model of diet-induced obesity. These findings highlight the positive effects of MR blockade on adipocyte dysfunction and insulin resistance.

### 6.1.4 Endothelial dysfunction

Obesity, insulin resistance and hypertension enhance endothelial dysfunction[296]. We could confirm these findings in obese mice and provide mechanistic insight by demonstrating that MR antagonism prevented endothelial dysfunction. This beneficial effect of MR antagonism might be either explained by indirect beneficial effects of EPL on the WAT, concerning inflammation and insulin resistance, or by direct EPL effects on the arterial vascular cells.

The indirect EPL-induced vascular protection in obesity could be due to 2 different beneficial actions of MR antagonism: First, due to restored glucose tolerance upon EPL treatment, since acute hyperglycemia reduces NO bioviability in the vasculature and enhances endothelial dysfunction[297-298]. Second, it could be explained by the decreased expression of adipokines in the WAT upon EPL treatment, since WAT-derived cytokines can contribute directly to the development of endothelial dysfunction[299-301]. In addition, type 2 diabetes has been shown to be associated with increased TNF-α, MCP-1, and IL-1b signaling, which exacerbates inflammation as well as superoxide production by NADPH oxidase in the vessel, thereby leading to endothelial dysfunction[302-304]. Thus, both the ameliorated glucose tolerance as well as diminished adipokine expression upon EPL treatment might be responsible for restored endothelial function in obesity.

On the other hand, there is accumulating evidence suggesting that the unfavorable effects of aldosterone on vascular function arise from a direct aldosterone-induced vasculopathy characterized by decreased NO and increased oxidative stress: aldosterone attenuates endothelial NO bioactivity via MR activation[171,305] and induces superoxide production via NADPH oxidase induction with further degradation of endogenous NO[171,240,250,306]. Moreover, aldosterone increases the expression of COX-2[266] and induces vascular inflammation[307]. These latter finding

would imply that aldosterone induces endothelial dysfunction directly via activation of MR in endothelial cells.

## 6.1.5 Aortic endothelial cell expression pattern

Endothelial cells line the luminal wall of the vessel and are therefore in direct contact with the circulating blood and the underlying vascular layers. Thus, they are in perfect position to receive signals from the circulation and mediate them to the vasculature. Since we could show profound metabolic changes in obesity, associated with increased plasma aldosterone levels and distinct MR-induced endothelial dysfunction, we decided to characterize the mRNA expression levels in isolated aortic endothelial cells. Intensive literature research revealed the lack of an efficient and quick methodology to isolate fresh aortic endothelial cells to investigate the direct effects of diet-feeding or hormone-administration on aortic endothelial cell expression. In turn, we decided to establish such an isolation protocol by ourselves, allowing us to characterize the influence of obesity and MR antagonism on aortic endothelial mRNA expression.

### 6.1.5.1 Inflammatory changes

In vascular inflammation, endothelial cells are characterized by the expression of various inflammatory genes such as ICAM-1, and VCAM-1[151]. Both are transmembrane cell adhesion molecules, involved in the adhesion and transmigration of leukocytes and monocytes into the tissue[308-310]. We could show that obesity induces the expression of VCAM-1 and ICAM-1 in aortic endothelial cells. In contrast, MR antagonism has no influence on VCAM-1 expression, whereby ICAM-1 expression is significantly attenuated by EPL.

It has been reported that ICAM-1 is regulated in response to oxidative stress and proinflammatory cytokines as IL1-β, TNF-α and IL-6[311-313]. Therefore, the beneficial effects of MR antagonism in obesity on ICAM-1 expression might be due to the attenuated expression of WAT-derived adipokines upon EPL treatment. In contrast to that, Caprio and colleagues[170] showed that aldosterone activates endogenous MR in cultured human coronary endothelial cells and promotes gene ICAM-1 expression and leukocyte adhesion. Since they used an considerable higher aldosterone concentration (3604.4ng/l) to induce aldosterone effects then we observe in the plasma of obese mice (416.7(±38.03)ng/l), we suggest that the MR-dependent ICAM-1 induction, observed by Caprio[170], might be due to the non-physiological aldosterone concentration.

On the other hand, various adipokines, secreted by the WAT, induce activation and inflammation in endothelial cells[22]. We could show that EPL attenuates MCP-1 and TNF-α expression in the WAT in obesity. Therefore, we suggest that the beneficial effects of MR antagonism on inflammatory molecules in aortic endothelial cells in obesity might be attributed to the EPL-induced advantageous regulation of adipokines in the WAT.

In obesity, the classic pathway of the complement system that is part of the innate immune system is massively activated in insulin resistant adipocytes[314]. The complement system consists of a number of proteins in the blood that circulated in an inactive precursor state. When stimulated by one of several triggers such as inflammation or infection, proteases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. The net result of this activation cascade is a potentiation of the response and activation of a cell-killing membrane attack complex[315]. The protease C1-inhibitor acts on the components of complement system and prevents its spontaneous activation. Also, the C1-inhibitor suppresses the influx of leukocytes to the site of inflammation by decreasing the vascular permeability[316]. A beneficial action C1-inhibitor has been demonstrated in several inflammatory diseases as endotoxin shock[317] and myocardial ischemia reperfusion injury[318]. In addition to the regulation of cell adhesion molecules, we proved that the C1-inhibitor of the complement system is beneficially regulated upon EPL treatment in obesity. We suggest that the attenuated inflammation in obesity by MR antagonism allows the expression of C1-inhibitor that contributes to anti-inflammatory changes in the circulation.

#### 6.1.5.2 ROS-related changes

ROS are key triggers of inflammation[319]. Their vascular levels are determined by the rate of superoxide production and degradation, depending on the expression and proper function of numerous enzymes involved in ROS handling by the cell. Systemic oxidative stress is known to be increased in obesity[320]. Along this line, we identified a pro-oxidative mRNA expression pattern in aortic endothelial cells obtained from obese mice, which may contribute to endothelial dysfunction. In terms of ROS scavenging enzyme systems, we detected attenuated levels of SOD-1 and SOD-3 in obese mice. This is in line with observations that obesity is associated with a decreased expression of SOD-3 in the WAT and the plasma[80]. Interestingly, we could show enhanced expression of these antioxidant enzymes upon EPL administration. Regarding ROS-generating enzymes, an increasing body of evidence suggests that the endothelial gp91phox-containing NADPH oxidase isoform (NOX2) is the most important superoxide anion source in the vascular wall[321]. Indeed, increased activity of NOX2 makes an important contribution to the pathogenesis of experimental models of vascular diseases cholesterol-induced atherosclerosis and hypertension[322-323]. Along that line, we showed that obesity enhances the expression of the NOX2 subunits p40phox and Rac-1. Interestingly, MR antagonism exerts beneficial down regulating effects not only on these two subunits, but also on p22phox, p47phox, and p40phox.

In summary, we observed a pronounced pro-oxidative mRNA expression pattern in aortic endothelial cells in obesity which was attenuated by MR antagonism. Therefore, some of these obesity-induced changes might be induced by the increased aldosterone in obesity.

### 6.1.5.3 Prostanoids and eNOS-related changes

The vascular expression of the prostanoids producing enzymes COX-1 and COX-2 is enhanced in various animal models of hypertension, diabetes and obesity, contributing to a pronounced endothelium-dependent vasoconstriction[133,324-328]. In hypertension, COX-1 co-localizes with prostacyclin synthase and induces the generation of prostacyclin[60]. This prostanoid is the major endothelium-derived contracting factor in the hypertensive rat aorta and mediates endothelial dysfunction[325,329-330]. In contrast, prostacyclin plays no major role in diabetes-associated endothelial dysfunction[331]. Instead, prostaglandin has been shown to be more important in carrying out vasoconstrictor actions in diabetic arteries[331]. Nevertheless, it is still not completely clear, which COX-derived prostanoids are the key players in obesity-induced endothelial dysfunction[332].

In agreement with the literature, mRNA analyses of endothelial cells revealed that obesity increases the expression of enzymes involved in prostanoid generation, such as COX-1 and prostacyclin synthase. Interestingly, pharmacological MR antagonism is not influencing any of these enzymes. Therefore, we propose that enhanced expression of prostanoid-producing enzymes in obesity is triggered by other stimuli than aldosterone-induced MR signaling. In hypercholesterolemia, COX-1 promotes vascular inflammation and thrombogenesis[333]. Since EPL is not changing plasma cholesterol levels in obesity[235], we suggest that high cholesterol levels are mainly responsible for enhanced COX-1 expression in obesity.

NO, generated by eNOS, plays an important role in vascular homeostasis, adhesion of inflammatory cells and function of platelets[334]. Three major factors may account for decreased NO levels, leading to endothelial dysfunction. First, functional abnormalities of the NO-synthase due to enzyme substrate or cofactor deficiency[335-336]; Second, decreased bioavailability of NO by oxidative stress[337] and third, reduced expression of eNOS. Several publications suggest that inflammation and expression of proinflammatory cytokines attenuate eNOS expression in endothelial cells in the vasculature[338-340]. Considering these three possibilities, we suggest that the non-significant increase in eNOS expression in aortic endothelial cells of obese mice might lead to an increased NO content in the aorta. Such an enhanced NO production in endothelial inflammation has been already described by others[341]. Since we showed an increased expression of NAPDH oxidase subunits in obesity as well, this might lead to an enhanced production of harmful peroxynitrite contributing to the generation of endothelial dysfunction in obesity. Additionally, we could show that EPL further enhances eNOS expression in endothelial cells. This might be due to anti-inflammatory changes associated with MR antagonism that increase eNOS expression. Since several NADPH oxidase subunits are decreased by EPL, oxidative stress might be attenuated and therefore NO bioavailability increased by MR antagonism. These changes might contribute to the restored endothelial function in obesity.

## 6.1.6 Conclusion

We could demonstrate that endothelial dysfunction in obesity correlates with impaired glucose tolerance and a proinflammatory expression profile in the white adipose tissue as well as in endothelial cells. The aortic endothelium in obesity is characterized by increased expression of proinflammatory adhesion molecules, NADPH oxidase subunits, prostanoid-generating enzymes and decreased antioxidant systems. Pharmacological MR blockade by EPL abolishes obesity-induced impaired glucose tolerance, WAT inflammation and endothelial activation. In aortic endothelial cells, pharmacological MR antagonism results in decreased expression of NADPH oxidase subunits as well as in enhanced expression of antioxidant systems and eNOS. Prostanoid-generating enzymes remain unaltered by EPL. Taken together, all advantageous effects, induced by MR antagonism in obesity, contribute to a beneficial modulation of endothelial mRNA expression, protecting the endothelium form dysfunctionality (Figure 41).

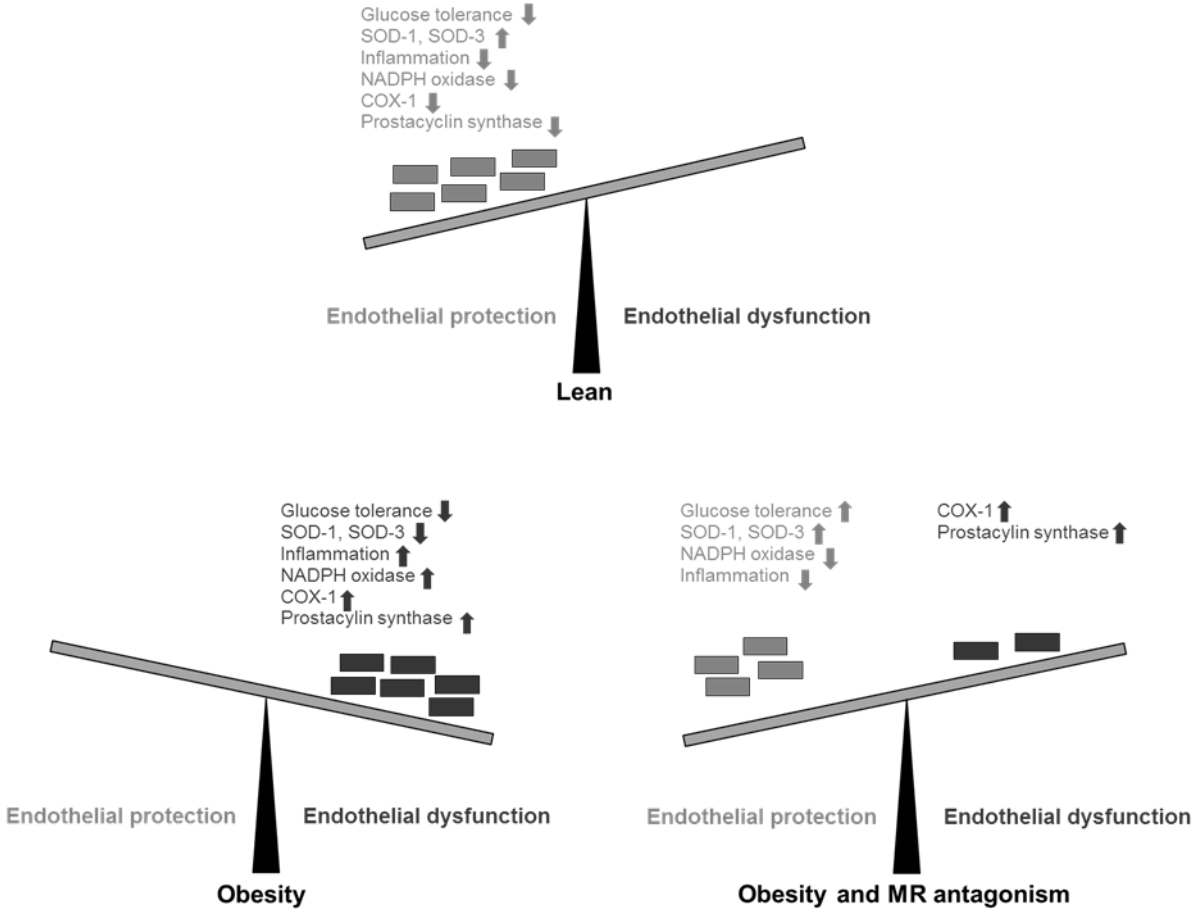


**Figure 41. Obesity-induced endothelial dysfunction is restored by MR antagonism.** EPL provides beneficial effects on glucose tolerance, inflammation and expression of ROS-generating enzymes, thereby diminishing endothelial dysfunction.

## 6.2 Endothelial MR ablation in obese or aldosterone-infused mice

### 6.2.1 Metabolic changes

In order to determine the role of the endothelial MR in the context of high endogenous (obesity) or high exogenous aldosterone-induced endothelial dysfunction we analyzed metabolic, inflammatory and vascular changes of obese or aldosterone-infused *EC MR KO* mice, respectively. We could show that a deficient endothelial MR signaling is not affecting weight or impairing glucose tolerance in obesity, ruling out an important role of the endothelial MR in the context of weight gain control or development of impaired glucose tolerance.

### 6.2.2 RAAS-related changes

In obese mice, we observed that plasma aldosterone levels are increased, in the presence or absence of the endothelial MR. Thus; endothelial MR-induced signaling pathways are not affecting the enhanced aldosterone secretion obesity. To characterize the role of increased aldosterone without obesity in detail, we infused lean *MR WT* and *EC MR KO* mice with exogenous aldosterone for 2 weeks. In order to mimic the aldosterone levels in obesity we infused 50 µg/kg/d aldosterone, resulting in an aldosterone plasma concentration of 413.5±36.01 ng/l. This is comparable to the plasma aldosterone concentration of obese *C57BL/6* (416.7±38.03 ng/l) and allowed us to study the role of aldosterone, independent of metabolic and inflammatory changes, associated with obesity. Since aldosterone was released by the osmotic minipumps without any circadian alterations, aldosterone-induced effects might be stronger in aldosterone-treated mice than in obese mice with endogenously increased aldosterone.

Unlike measured in obese *C57BL/6* mice (Figure 23), no increase in tissue MR expression was observed in obese *EC MR KO* mice and their corresponding *WT* littermates. Since the increase in MR expression is not pronounced in obese *C57BL/6* either, the change in tissue MR expression is in our mouse model of diet-induced obesity is likely negligible.

### 6.2.3 White adipose tissue inflammation

White adipose tissue contains multiple cells including preadipocytes, adipocytes, small blood vessels, and macrophages. Cross-talk between the different adipose tissue cell types, in particular between macrophages, preadipocytes, and adipocytes, is thought to be an important factor for promoting a proinflammatory state in obesity[30,342]. Especially macrophages are important cytokine-secreting cells in the obese WAT[54]. As also shown by others[289] we observed

that obesity induces a pronounced inflammation in the WAT of mice. In addition, we demonstrated that expression of MCP-1, TNF-α as well as of macrophage marker CD68, is induced to the same extent in obese *EC MR KO* and *MR WT* mice, suggesting that endothelial MR signaling is not affecting inflammation in the obese WAT.

Recently, it has been demonstrated that a conditional MR ablation in myeloid cells, that are macrophage progenitor cells, attenuates MCP-1 release in macrophages and diminishes overall inflammation in cerebral ischemia[343]. Along this line, we observed both, a successful endothelial MR ablation and an additional MR deletion in macrophages isolated from *EC MR KO* mice. As the obesity-induced inflammation in the WAT of *EC MR KO* mice remains unaltered compared with *MR WT* mice, we propose that the impact of the unspecific MR ablation in macrophages is negligible in our setting.

Aldosterone has been reported *in vitro* to increase the expression of MCP-1, TNF-α, and IL-6 in cultured preadipocytes[219]. In contrast, we showed *in vivo* that an exogenous aldosterone infusion does not enhance the expression of TNF-α, MCP-1 or macrophage accumulation in the WAT. This observation might due to the expression of 11β-HSD1 in the WAT tissue[17]. Since 11β-HSD1 determines intracellular glucocorticoid levels by regenerating cortisol from cortisone[173], the MR in the adipose tissue is likely to be more activated by cortisol than by aldosterone[217], even if aldosterone levels are increased. Furthermore, activity and expression of 11β-HSD1 are known to be increased in obese WAT[212-213] and are associated with the development of metabolic syndrome[214-215,344]. Accordingly, it is proposed that in obesity it is the increased intracellular level of glucocorticoids rather than of aldosterone that triggers MR-induced adipocyte inflammation.

### 6.2.4 Endothelial function

As described above, MR antagonism restores obesity-induced endothelial dysfunction completely. To elucidate the role of the endothelial MR in this context, we analyzed *EC MR KO* mice with regard to endothelial function. As we did not observe any impact of endothelial MR ablation on body weight, glucose tolerance, or adipose inflammation in obesity, we were able to elucidate the role of the endothelial MR independent, of other factors favoring endothelial dysfunction.

In the course of our experiments, we could emphasize the central role of the MR in endothelial cells: Deletion of the endothelial MR is sufficient to abolish obesity-induced as well as aldosterone-induced endothelial dysfunction. Consequently, the endothelial MR is critical for endothelial dysfunction in obesity.

Considering our observation that expressions of COX-1 and prostacyclin synthase are increased in aortic endothelial cells in obesity, we assessed the role of COX in aldosterone-induced endothelial dysfunction by using the COX-inhibitor indomethacin. Interestingly, we could show that COX-blockade restores endothelial function, underlining the importance of prostanoids in obesity- and aldosterone-induced endothelial dysfunction. The role of prostanoids in aldosterone-induced endothelial function has been described in hypertensive and normotensive rats[239]. Furthermore, Traupe and collegues[328] demonstrated that obesity induces endothelial dysfunction in a COX-1 dependent manner and enhances the expression of the vascular TP. These changes induce vasoconstriction in obesity.

Taken together, these results suggest that the endothelial MR as well as vasoconstrictor prostanoids contribute to both obesity- and aldosterone-induced endothelial dysfunction.

## 6.2.5 Aortic endothelial cell expression pattern

The previous observations, allowed us to characterize the influence of a pharmacological MR blockade on aortic endothelial cells in obesity - reflecting a state of endogenous aldosterone elevation. To further characterize, which ones of these changes are mediated via the endothelial MR, we analyzed aortic endothelial cells of *EC MR KO* and *MR WT* mice treated with exogenous aldosterone. Our data revealed changes concerning ROS-producing and -degrading enzyme systems, as well as concerning enzymes, involved in the generation of vasoconstrictor prostanoids.

### 6.2.5.1 Inflammatory changes

We observed endogenous expression of ICAM-1 and VCAM-1 in lean *C57BL/6* mice, and an increased endothelial activation in obesity. Interestingly, exogenous aldosterone administration alone – in the absence of obesity – is not sufficient to increase expression of adhesion molecules. Thus, we conclude that aldosterone is not inducing these obesity-associated proinflammatory changes in aortic endothelial cells.

### 6.2.5.2 ROS-related changes

We showed that obesity is associated with a suppressed expression of SOD-1 and SOD-3. Interestingly, we observed a similar expression profile of these antioxidant systems upon aldosterone administration. Whereas MR antagonism restores the expression of both enzymes in obesity, endothelial MR ablation in aldosterone-treated mice ameliorates only the expression of SOD-1. Thus, in obesity, aldosterone decreases SOD-1 expression in an endothelial MR-dependent manner. In contrast, both endogenous (obesity) as well as exogenous aldosterone seem to attenuate SOD-3 expression, independently of the endothelial MR in aortic endothelial

cells. The enhanced SOD-3 expression in obesity upon MR antagonism might be due to endothelial MR independent anti-inflammatory effects of EPL in obesity.

Moreover, we could demonstrate an advantageous decrease of NADPH oxidase subunit expression in obesity upon MR antagonism. It has been demonstrated in the literature, that aldosterone increases the aortic expression of p22phox, gp91phox, p47phox and Rac-1[253-254,345]. Along that line, we could show that p22phox is regulated by aldosterone in an endothelial MR-dependent manner. Interestingly, p22phox is induced by exogenous aldosterone but not by obesity. This might be due to the fact that the endogenous aldosterone is released in a circadian rhythm, whereas exogenous aldosterone is administered by constant infusion, having a greater impact on the endothelial MR. Nevertheless, the attenuated p22phox expression upon EPL in obesity might be due to blocked endothelial MR signaling.

Since we demonstrated that only p22phox is regulated by exogenous aldosterone endothelial MR-dependently, we presume that all other subunits of the NADPH oxidase, modulated by obesity and MR antagonism, are regulated by EC MR-independent pathways.

Taken together, we observed that aldosterone decreases both SOD-1 as well as SOD-3 and enhances p22phox expression in aortic endothelial cells. Of note, only SOD-1 and p22phox are regulated in similar directions by MR antagonism and endothelial MR ablation, respectively. Thus, we propose that these two mediators are modulated by the endothelial MR in obesity and are key factors in the generation of oxidative stress in this setting.

#### 6.2.5.3 Prostanoids related changes

We demonstrated that aldosterone induces COX-1 and prostacyclin synthase expression in aortic endothelial cells. Our findings are in agreement with the literature[346-347] and match our organ chamber experiments.

Interestingly, COX-1 expression is induced by aldosterone in an endothelial MR-dependent manner, whereas MR antagonism does not prevent obesity-induced COX-1 increase. We suggest that the endothelial MR plays an important role in inducing COX-1 expression when aldosterone is chronically increased by exogenous administration. In obesity, in contrast, other factors besides elevated aldosterone participate to the induction of COX-1 mRNA, in particular also hypercholesterolemia[333]. Prostacyclin is a potent vasoconstrictor and plays a major role in aldosterone-induced endothelial dysfunction in normotensive and hypertensive conditions[346-347]. Along that line, we could demonstrate that aldosterone induces prostacyclin synthase expression in an endothelial MR-independent manner. Taken together, we showed that in obesity, aldosterone is of minor importance in inducing COX-1 expression, whereas aldosterone is responsible for prostacyclin synthase induction in an endothelial MR independent manner.

Concerning eNOS expression, we did not observe any expression changes upon aldosterone treatment. We suggest, that eNOS is not regulated by aldosterone directly.

### 6.2.6 Conclusions

In conclusion, our work with genetically ablated MR in endothelial cells identifies the endothelial MR as a crucial mediator in obesity- and aldosterone-induced endothelial dysfunction. Prostanoids play an important role in the development of endothelial dysfunction in this pathophysiological setting, since COX-inhibition restores aldosterone-induced endothelial dysfunction. In fact, aldosterone enhances expression of COX-1 in an endothelial MR-dependent way and prostacyclin synthase expression in an endothelial MR-independent way. Nevertheless, endothelial MR-induced COX-1 activation seems to be of minor importance in obese mice; in contrast, aldosterone-induced prostacyclin synthase expression could be responsible for increased prostacyclin levels in obesity. Furthermore, aldosterone modulates NADPH oxidase subunit p22phox and SOD-1 in a MR-dependent manner. Thus, endothelial MR signaling in obesity plays a critical role in generation of oxidative stress.

Taken together, we propose that in obesity, endogenous aldosterone enhances production of prostanoids. The endothelial MR-mediated alterations concerning ROS-producing enzymes might be crucial in the generation of endothelial dysfunction (Figure 42).

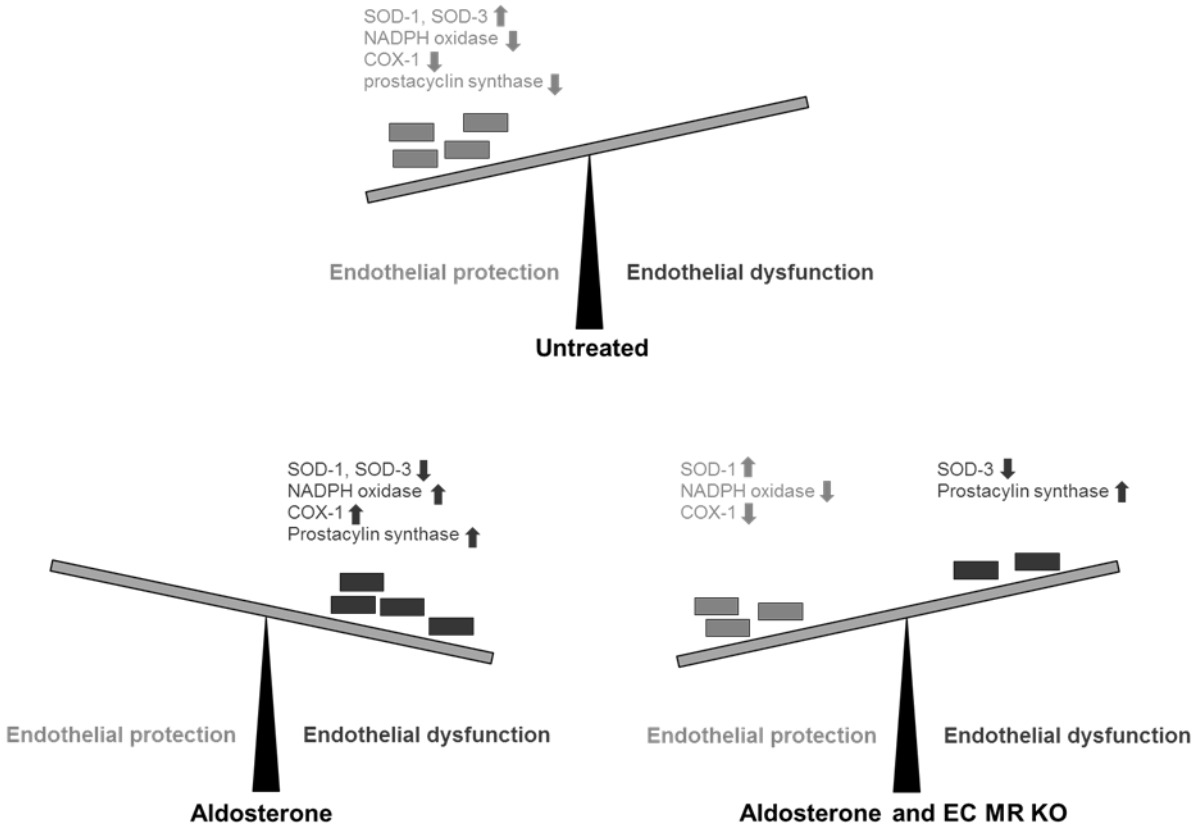


**Figure 42. Aldosterone-induced endothelial dysfunction is restored by endothelial MR deletion.** Endothelial MR ablation provides beneficial effects on ROS-and prostanoids-generating enzymes, thereby diminishing endothelial dysfunction.

## 6.3 Summary: Aldosterone effects in obesity-induced endothelial dysfunction

In this thesis, we could show that aldosterone and the endothelial MR play an important role in the development of obesity-induced endothelial dysfunction.

Obesity is associated with impaired glucose tolerance as well as adipose tissue inflammation. Moreover, obesity has a profound influence on aortic endothelial cell expression concerning inflammatory molecules, antioxidant systems, NADPH oxidase and enzymes involved in the generation of prostanoids. All of these effects contribute to the generation of endothelial dysfunction. Interestingly, MR antagonism attenuates most of the factors favoring endothelial dysfunction and induces the expression of eNOS. Only obesity-induced expression of COX-1 and prostacyclin synthase is not beneficially influenced by MR antagonism. Nevertheless, the advantageous effects of MR antagonism are sufficient to restore obesity-induced endothelial dysfunction.

Analyses of mice lacking the endothelial MR revealed that endothelial MR ablation restores obesity- as well as aldosterone-induced endothelial dysfunction, without affecting glucose tolerance or white adipose tissue inflammation. Interestingly, we could show that aldosterone induces expression of the NADPH oxidase subunit p22phox and decreases expression of the antioxidant system SOD-1 in a MR-dependent manner in aortic endothelial cells. This might increase oxidative stress that contributes to endothelial dysfunction.

Both aldosterone and obesity induce COX-1 expression in aortic endothelial cells. MR antagonism does not alter COX-1 expression in obesity, whereas MR ablation in endothelial cells ameliorates aldosterone-induced COX-1 expression. We presume that besides endothelial MR-mediated COX-1 induction, COX-1 expression in obesity can be induced by other pathophysiological changes such as hypercholesterolemia. Endothelial MR-induced COX-1 expression might be of minor importance in obesity. Prostacyclin synthase expression is increased both in obesity as well as upon exogenous aldosterone administration, either. Since the increased prostacyclin synthase is not affected by EPL or endothelial MR ablation, we propose this enzyme to be induced by aldosterone in an endothelial MR-independent manner.

Altogether, we identified the following factors to be involved in obesity-induced endothelial dysfunction. First, endogenous aldosterone in obesity enhances oxidative stress by altering ROS-related proteins via the endothelial MR. Second, endogenous aldosterone in obesity regulates prostacyclin synthase expression in an endothelial MR-independent manner. Third, obesity enhances expression of COX-1 via an endothelial MR-independent mechanism and, of minor importance, via an endothelial MR-dependent mechanism (Figure 43).

The endothelial MR plays a central role in regulating the expression of NADPH oxidase subunit p22phox and antioxidant SOD-1. Indeed, a beneficial regulation of theses enzymes in obesity tips the balance to the anti-oxidative side, resulting in attenuated endothelial dysfunction.

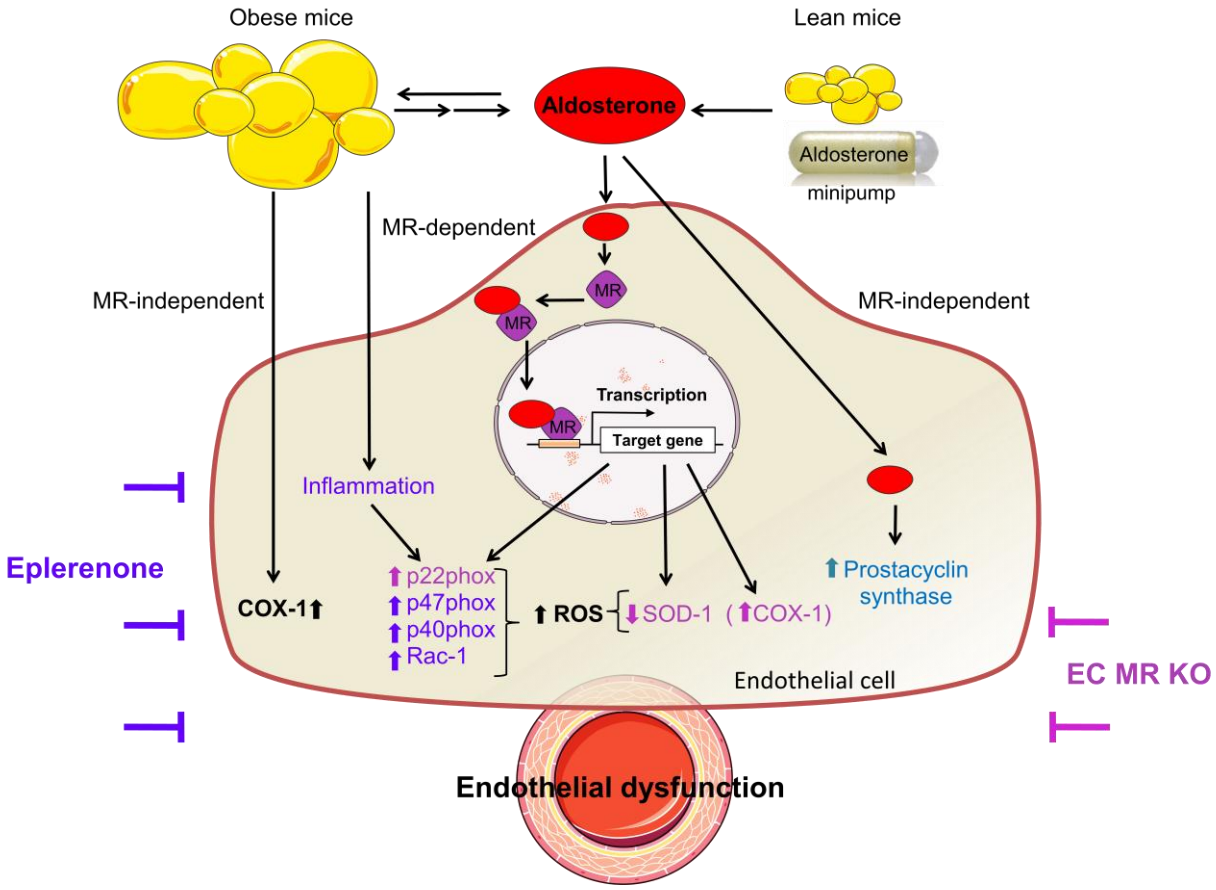


**Figure 43. Mechanisms of aldosterone-induced endothelial dysfunction in obesity**. Pathophysiological changes in obesity contribute to endothelial function and are not mediated via the endothelial MR, but can be blocked by EPL (■). Some of the aldosterone-induced changes contributing to endothelial dysfunction are mediated via the endothelial MR (■) and others independently of the endothelial MR (■).

The expression of ROS- and prostanoid-generating enzymes is increased in obesity and contributes to endothelial dysfunction. The question rises how the endothelial MR-dependent oxidative stress is key player in obesity-induced endothelial dysfunction.

First, endothelial MR-mediated changes in oxidative stress might directly damage endothelial cells[348] by decreasing the content of vasodilating NO in the vasulature[111].

Second, ROS and prostanoids are able to induce each other's expression, promoting a viscous cycle that enhances endothelial dysfunction in obesity. On one side, COX-derived prostanoids can enhance generation of ROS in the endothelium and thereby impair endothelium-dependent contraction[349]. On the other side, ROS activate COX and induce prostacyclin production in cultured endothelial cells[350]. This positive feedback loop in endothelial cells could lead to an amplification of prostanoid production and to increased oxidative stress in endothelial cells. Moreover, increased extracellular oxidative stress, due to increased endothelial NADPH oxidase, can induce vasoconstriction as it does in hypertension by acting on COX in smooth muscle cells[351]. Along that line, ROS induce VSMC contraction by stimulating $Ca^{2+}$ release from the sarcoplasmatic reticulum[352] and ameliorates the stability of the prostanoid receptors at the cell membrane of VSMCs *in vitro*[353-354]. This is in line with the observation, that TP expression is increased in atherosclerotic lesions in mice and humans[355-356].

Taken together, the increase in oxidative stress as well as the increase of prostanoids production in aortic endothelial cells might lead to a vicious circle, in which the harmful effects of these mediators amplify each other, thereby enhancing the progression of endothelial dysfunction. The increased expression of p22phox in obesity mediated by the endothelial MR- might be one of the crucial mediators that tips the balance in the generation of endothelial dysfunction in obesity.

# 6.4 Future directions

## 6.4.1 Eplerenone - a potent anti-inflammatory molecule

Many studies, including our own, demonstrate that MR blockade exhibits anti-inflammatory properties in the animal model[219,234-235]. These studies propose MR antagonism as a promising tool for treating obesity-associated inflammatory diseases such as endothelial dysfunction and type 2 diabetes.

While the treatment of endothelial dysfunction in obesity appears reasonable and safe, treatment of type 2 diabetes, the most common cause of chronic kidney failure[357], needs to be approached with caution. Kidney failure may favor hyperkalemia, given the central role of the kidney and aldosterone in plasma potassium homeostasis. Thus, MR blockade has to be used carefully in chronic kidney disease given the increased risk of hyperkalemia.

## 6.4.2 Targeting the endothelial MR - a new strategy to treat obesity-induced endothelial dysfunction and vascular disease

The present thesis demonstrates for the first time that endothelial MR plays a crucial role in obesity-induced endothelial dysfunction. Therefore, the endothelial MR could be an interesting pharmacological target to treat endothelial dysfunction, impaired glucose tolerance, and pro-inflammatory changes in obese patients without significant kidney disease.

Whereas we could show that aldosterone induces prostacyclin synthase in an endothelial MR-independent manner, COX-1 regulation in obesity remains to be clarified. COX-1 expression was unaltered in obesity upon EPL treatment, and abolished in aldosterone-treated mice, when the endothelial MR was ablated. To characterize the role of the endothelial MR in this context further, aortic endothelial cell analyses of obese *EC MR KO* mice would be of interest. Such analyses disclose the endothelial MR-dependent pathways in obesity in more detail and are the missing puzzle in our characterization of aortic endothelial cells. Along that line, it would be appealing to investigate if a selective COX-1 or prostacyclin synthase inhibition is sufficient to restore obesity- or aldosterone-induced-endothelial dysfunction.

Since we could show a profound impact of obesity and aldosterone on enzyme systems involved in ROS- as well as prostanoid-generation, measuring the actual content of ROS and prostanoids in the vasculature of the different mice seems to be interesting.

One limitation of our study is that our data do not allow us to conclude whether the protective effects concerning endothelial function seen in *EC MR KO* mice are exclusively due to an endothelial MR ablation. As reported above, adipose tissue macrophages and T cells are the main cytokine-secreting cells in the white adipose tissue in obesity [55]. Of note, we observed no

changes in proinflammatory expression pattern in the WAT of obese *EC MR KO* mice compared with *EC MR WT* mice. Thus, partial ablation of the MR in adipose tissue macrophages may play a minor role in the context of obesity-induced endothelial dysfunction. Nevertheless, future studies with a more specific endothelial cell promoter to ablate the MR, using the CreLoxP system would be meaningful.

We were able to show that the endothelial MR plays a crucial role in endothelial dysfunction, being the initial step of atherogenesis. In addition to the growing evidence related to the protective effects of the MR antagonists in the metabolic syndrome, our results highlight the importance of the relationship between aldosterone and endothelial MR in the development of this disease.

# 7 List of figures

# 8 Abbreviations

| | |
|---|---|
| ACE | angiotensin-converting-enzyme |
| Ach | acetylcholine |
| ACTH | adrenocorticotropic hormone |
| AngI | angiotensin I |
| AngII | Angiotensin II |
| AP-1 | activator protein-1 |
| ARB | angiotensin-receptor blockers |
| ARF | aldosterone-releasing factor |
| $AT_1$ | type I AngII receptor |
| $AT_2$ | type II AngII receptor |
| BAT | brown adipose tissue |
| BH4 | 4-tetrahdrobiopterin |
| bp | base pairs |
| BSA | bovine serum albumine |
| C1-inhibitor | inhibitor of the complement factor 1 |
| $CaCl_2$ | calcium chloride |
| CD86 | macrophage glycoprotein |
| cGMP | cyclic guanosin monophosphate |
| CNS | central nervous system |
| COX-1 | cyclooxygenase-1 |

| | |
|---|---|
| COX-2 | cyclooxygenase-2 |
| CREM | cyclic AMP-response element modulator |
| CRP | C-reactive protein |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| *EC MR KO* | *MRflox//Tie2Cre* |
| EDCF | Endothelium-derived contracting factors |
| EDRF | endothelium-derived relaxing factors |
| EKODE | 12,13-epoxy-9-keto-10(*trans*)-octadecenoicacid |
| ENaC | amiloride-sensitive epithelial sodium channel |
| eNOS | endothelial nitric oxide synthase |
| EPL | eplerenone |
| FFA | free fatty acids |
| FITC | fluoresceinisothiocyanat |
| fwd | forward |
| G6P-DH | glucose-6-phosphate dehydrogenase |
| GC | guanylylcyclase |
| GLUT | glucose transporter |
| GR | glucocorticoid receptor |
| Hbb-b1 | hemoglobin, beta adult major chain |
| HFD | high-fat diet |
| HFD EPL | high-fat diet containing eplerenone |

| | |
|---|---|
| ICAM-1 | intercellular adhesion molecule-1 |
| IL | interleukin |
| IP | prostacyclin receptor |
| $IP_3$ | inositol 1,4,5-trisphosphate |
| IRS | insulin receptor substrate |
| JNK | c-Jun N-terminal kinases |
| KCl | kalium chloride |
| KO | knockout |
| LDL | low-density lipoprotein |
| LOX-1 | oxidized LDL receptor |
| MCP-1 | monocyte chemoattractant protein-1 |
| $MgCl_2$ | magnesium chloride |
| min | minutes |
| MMP | matrix metalloproteinase |
| MR | mineralocorticoid receptor |
| mRNA | messenger ribonucleic acid |
| *MR WT* | *MRflox/WT* |
| NaCl | natrium chloride |
| NADPH | nicotinamide adenine dinucleotide phosphate-oxidase |
| $NaHCO_3$ | sodium bicarbonate |
| $NaH_2PO_4$ | sodium phosphate |
| $Na_2SO_4$ | sodium sulfate |
| ND | normal diet |
| NE | norepinephrine |
| Nedd4 | neuronal precursor cell expressed developmentally downregulated 4 |
| nedd4-2 | nedd4 isoform 2 |
| NF-kB | nuclear factor 'kappa-light-chain-enhancer' of activated B-cells |

| | |
|---|---|
| NO | nitric oxide |
| NOS | NO synthase |
| NOX | NADPH oxidase |
| $ONOO^{\cdot-}$ | peroxynitrit |
| PAI-1 | plasminogen activator inhibitor-1 |
| PBS | phosphate buffered saline |
| $PGE_2$ | prostaglandin $E_2$ |
| $PGD_2$ | prostaglandin $D_2$ |
| $PGH_2$ | prostaglandine |
| $PGI_2$ | prostacyclin |
| *$PLA_2$* | phospholipase 2 |
| PKG | cGMP-dependent protein kinases |
| PPAR-y | peroxisome proliferator-activated receptor-y |
| RAAS | renin-angiotensin-aldosterone-system |
| ROS | reactive oxygen species |
| rev | reverse |
| rpm | rounds per minute |
| RT-PCR | real time reverse transcription polymerase chain reaction |
| sec | seconds |
| SEM | standard error of the mean |
| sGC | soluble guanylatecyclase |
| SGK1 | serum-and glucocorticoid-induced kinase 1 |
| SNP | sodium nitroprussid |
| SOD | superoxide dismutases |
| SRE | steroid response element |
| TLCK | Nα-Tosyl-L-lysinchlormethylketon - Hydrochlorid |
| TNF-α | tumor necrosis factor-α |

| | |
|---|---|
| TP | prostanoid receptor/ thromboxane receptor |
| $TXA_2$ | thromboxane |
| U | Units |
| VCAM-1 | vascular cell adhesion protein-1 |
| VSMC | vascular smooth muscle cells |
| vWF | von Willebrandt-factor |
| WAT | white adipose tissue |
| 11β-HSD | 11β-hydroxysteroid dehydrogenase |

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# 10 Curriculum Vitae

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|---|---|
| Name | SCHÄEFER |
| Firstname | Nicola |
| Date of Birth | 20.01.1982 |
| Citizen of | Germany |

| | |
|---|---|
| 04/2007-date | PhD study in Integrative Molecular Medicine, Institute of Physiology, University of Zurich, in Zurich, Switzerland (supervisors: Prof. Dr. François Verrey, PD Dr. Christian M. Matter, Prof. Dr. C. Wagner, Prof. Dr. Thierry Pedrazzini) |
| 02/2007 | Certified Biologist (Grade 1,6) |
| 03/ 2006 – 12/2006 | Diploma thesis at the German Heart Center, Experimental Children Cardiology, Clinics of the Technical University Munich, Germany<br>Titel: Role of G-protein coupled receptors in the generation of reactive oxygen species (supervisors: PD Dr. Agnes Görlach, Prof. Dr. Albrecht Wendel) |
| 10/2001 - 11/2006 | Biology, University of Constance, Germany |
| 07/2001 | Certificate: Abitur |
| 09/1992 – 07/2001 | Windeck-Gymnasium Bühl, Germany |
| 09/1988 – 07/1992 | Grundschule Greffern, Germany |

**Rewards**

| | |
|---|---|
| 09/2009 | Asher-Hess Competition, Swiss Physiological Society, in Bern, Switzerland<br>Prize for oral presentation |

**Unpublished manuscripts**

**N Schäfer**, C Lohmann, A Vergopoulos, F Ruschitzka J Nussberger,T F Lüscher, F Verrey, C M. Matter. Endothelial mineralocorticoid receptor confers oxidative stress and endothelial dysfunction in diet-induced obesity

**Contribution to publications**

Besler C, Heinrich K, Rohrer L, Doerries C, Riwanto M, Shih DM, Chroni A, Yonekawa K, Stein S, **Schaefer N**, Mueller M, Akhmedov A, Daniil G, Manes C, Templin C, Wyss C, Maier W, Tanner FC, Matter CM, Corti R, Furlong C, Lusis AJ, von Eckardstein A, Fogelman AM, Lüscher TF, Landmesser U. Mechanisms underlying adverse effects of HDL on eNOS-activating pathways in patients with coronary artery disease. J Clin Invest. 2011 Jun 23. [Epub ahead of print]

Aortic endothelial cell isolation

Winnik S, Lohmann C, Richter EK, **Schäfer N**, Song WL, Leiber F, Mocharla P, Hofmann J, Klingenberg R, Borén J, Becher B, Fitzgerald GA, Lüscher TF, Matter CM, Beer JH. Dietary alpha-linolenic acid diminishes experimental atherogenesis and restricts T cell-driven inflammation. Eur Heart J. 2011 Feb 9. [Epub ahead of print]

Vascular function analysis

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Tissue harvesting and processing

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Tissue harvesting and processing

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